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EXPRESSED GENES THAT DEFINE THE OSTEOCLAST PHENOTYPE

RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 60/432,700, filed December 11, 2002, the entire contents of which is hereby incorporated by reference.

GOVERNMENT SUPPORT

The invention was supported, in whole or in part, by grant DE-07378 from the National Institutes of Health. The Government has certain rights in the invention.

BACKGROUND OF THE INVENTION

Bone production by osteoblasts and bone resorption by osteoclasts is critical for normal bone development and remodeling. Excessive resorption is a key pathogenic component in osteopenic conditions such as osteoporosis, arthritis, periodontitis and certain malignancies. Bone resorption is regulated by a complex system of hormones and locally-produced cytokines that stimulate osteoblasts and stromal cells to express Receptor Activator of NF-κB Ligand (RANKL), which results in the differentiation and activation of osteoclasts. The processes by which osteoclast differentiation and activation occur are currently not well understood.

Additional intracellular signaling pathways may also be important regulators of osteoclastic resorption and bone production by osteoblasts. The identification of the molecules that mediate differentiation and activation of osteoclasts and production of bone by osteoblasts may lead to new methods for regulating bone resorption and bone mineral density, and to new treatments for osteopenic conditions.

SUMMARY OF THE INVENTION

The invention features methods of inhibiting osteoclast-mediated bone resorption by inhibiting activity of a gene product encoded by an osteoclast associated gene. Examples of osteoclast associated genes include OC 1-285, *brn3a*, *brn 3b*, and *brn3c*. Also included are methods of inhibiting osteoclast-mediated bone resorption by inhibiting expression of an

osteoclast associated gene. The osteoclast associated genes encode gene products that include, for example, MIP1γ, Brn3a, Brn3b, and Brn3c.

The invention includes methods of inhibiting osteoclastogenesis by contacting an osteoclast precursor cell with an inhibitor of MIP1γ. Examples of precursor cells are monocytes and macrophages. Inhibitors include an antibody that binds to an epitope of MIP1γ. One example of an epitope is a CCR1 receptor binding domain. Inhibitors are also polypeptides that bind to a CCR1 receptor but do not activate the receptor. Inhibitors also do not induce or increase the production of MIP1γ.

The invention also involves methods of promoting osteoclast survival by contacting an osteoclast cell with a MIP1γ polypeptide resulting in a decrease in apoptotic cell death in the presence of the polypeptide compared to that in the absence of the polypeptide. Other agents or compounds for promoting osteoclast survival include RANKL, LPS and IL-1α.

The invention further includes methods of inhibiting proliferation of osteoclast cells by contacting the cells with an inhibitor of MIP1γ expression or activity.

Also described herein are methods of stimulating osteoclast-mediated bone resorption by contacting an osteoclast cell with a MIP1γ polypeptide.

The invention also includes methods of inhibiting osteoclastogenesis by contacting an osteoclast precursor cell with an inhibitor of an activity of a gene product of an osteoclast associated gene. One method for inhibiting osteoclastogenesis is to inhibit the fusion of precursor cells thereby inhibiting the formation of osteoclastic giant cells. The inhibition of fusion of a plurality of precursor cells into an osteoclastic giant cell is inhibited by at least 10%, 20%, 40%, 50%, 60%, 75%, 90%, 95%, or greater. In one example the inhibitor is a polynucleotide that is a decoy oligonucleotide. A decoy oligonucleotide is identical to or similar to the binding site or target site of a POU domain protein, such as the Brn3 family of proteins. Decoy oligonucleotides inhibit binding to endogenous target sites. The inhibitors, including decoy oligonucleotides, inhibit binding of a Brn3 polypeptide to a target site.

The invention also involves methods of inhibiting bone resorption by increasing activity of a gene product of an osteoclast associated gene, such as OC 286–364. Other methods of inhibiting bone resorption involve increasing expression of an osteoclast associated gene, such as OC 286–364.

The invention also includes a reference expression profile. An expression profile includes a pattern of gene expression of two or more genes selected from the group consisting of OC1-364.

OSTEOCLAST MARKER – is a nucleic acid or polypeptide which is differentially expressed in an mature osteoclast cell compared to a non-osteoclast cell or an osteoclast precursor cell. For example, the level of expression of an osteoclast marker (e.g., OC1-285, Brn3a, b, c) is preferentially expressed in osteoclasts. The level of expression of OC1-285 is a least 10%, 20%, 50%, 100% or 200% or greater in osteoclasts compared to non-osteoclast cells. Non-osteoclast cells include monocytes, macrophages, and osteoblasts. The level of expression of a nucleic acid and/or polypeptide is measured using methods known in the art, e.g., RT-PCR and antibody-based immunochemical assays, respectively. Alternatively, the level of expression is reduced by at least 10%, 20%, 50%, 100% or 200% or greater in osteoclasts compared to non-osteoclast cells, as in the case with OC286-364.

The invention further includes methods for determining whether a subject is suffering from or is predisposed to developing a bone disease by providing a biological sample from the subject; detecting at least one osteoclast marker in the biological sample; measuring the level of expression of said at least one osteoclast marker; and comparing the level of expression of said osteoclast marker in said biological sample to the level of expression of the osteoclast marker in a control sample. An alteration in the level of expression of the osteoclast marker in the subject compared to the control sample indicates the presence of or predisposition to a bone disease. An increase in the level of expression of at least one osteoclast marker, OC 1-285, Brn3a, Brn3b, Brn3c, MIP-1γ, CCR1, MIP-1α, or RANTES or combinations thereof (for example, in an expression profile), compared to the control sample indicates the presence of or predisposition to a bone disease. A decrease in the level of expression of at least one osteoclast marker, OC 286-364 or combinations thereof (for example, in an expression profile), compared to the control sample indicates the presence of or predisposition to a bone disease.

Bone resorption diseases include osteoporosis, hyperparathyroidism, Paget's disease, inflammatory conditions, rheumatoid arthritis, osteoarthritis, and periodontitis. Bone generating diseases include osteopetrosis, axial osteosclerosis, and Osteopathia striata.

Also described herein are methods for identifying a therapeutic agent for use in treatment of a bone disease by providing a biological sample expressing an osteoclast marker; contacting the cell with a composition comprising a candidate substance; and measuring the level of expression of an osteoclast marker. A change in the level of expression of at least one osteoclast marker or a change in the expression profile of two or more osteoclast markers in the presence of the substance compared to the expression of said osteoclast marker in the absence of the substance, identifies the candidate substance as a potential therapeutic agent.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-1E are a list of the genes expressed at higher levels in RAW 264.7 macrophage-like cells stimulated with Receptor Activator of NFκB Ligand (RANKL) and normal mouse bone marrow macrophages stimulated with Macrophage-Colony Stimulating Factor (M-CSF) and RANKL, compared to unstimulated cells (control). "I" in the "Change" columns means that expression of the indicated gene is increased upon stimulation compared to control samples. The first column marked "Change" refers to samples of normal bone marrow cells and the second column marked "Change" refers to samples of RAW264.7 cells. "Status" refers to the net result (increased expression) of the indicated gene. "Change p value" is calculated by Affymetrix software for normal bone marrow cells and for RAW264.7 cells. "Average" refers to the average p values of the two samples.

Figures 2A-2B are a list of the genes expressed at lower levels in RAW 264.7 macrophage-like cells stimulated with Receptor Activator of NFκB Ligand (RANKL) and normal mouse bone marrow macrophages stimulated with Macrophage-Colony Stimulating Factor (M-CSF) and RANKL, compared to unstimulated cells (control). "D" in the "Change" columns means that expression of the indicated gene is decreased upon stimulation compared to control samples. The first column marked "Change" refers to samples of normal bone marrow cells and the second column marked "Change" refers to samples of RAW264.7 cells. "Status" refers to the net result (decreased expression) of the indicated gene. "Change p value" is calculated by Affymetrix software for normal bone marrow cells and for RAW264.7 cells. "Average" refers to the average p values of the two samples.

Figures 3A-3D are bar graphs of the kinetics of chemokine production by RANKL-stimulated RAW264.7 and bone marrow cells. (A) RAW264.7 cells were stimulated with RANKL (10 ng/ml) for 5 days. (B) Bone marrow cells were stimulated with RANKL (20 ng/ml) and M-CSF (50 ng/ml) for 7 days. Chemokine levels were measured by ELISA. Results represent the mean ± SD of triplicate cultures .

Figures 4A-4B are bar graphs of the effect of exogenous MIP-1γ on RANKL-induced osteoclast differentiation. (A) RAW264.7 cells were stimulated for 5 days with the indicated doses of MIP-1γ, in the presence or absence of RANKL (10 ng/ml). (B) Bone marrow cells were stimulated for 7 days with MIP-1γ in the presence or absence of RANKL (20 ng/ml) and M-CSF (50 ng/ml). The culture medium was supplemented with (hatched columns) or without (open columns) 1 μg/ml of polymyxin B to block any contaminating LPS. TRAP-positive cells with >3 nuclei were counted as osteoclasts. The results shown are the mean ± SD of three independent experiments.

Figures 5A-5B are are graphs of the anti-MIP-1γ antibody reduction of RANKL-induced osteoclast differentiation. (A) RAW264.7 cells were cultured with RANKL (10 ng/ml) and neutralizing anti-MIP-1γ antibody or an unreactive control IgG1 antibody for 5 days. (B) Bone marrow cells were cultured in the presence of RANKL (20 ng/ml) and M-CSF (50 ng/ml), in the presence of neutralizing anti-MIP-1γ antibody or unreactive control IgG_1 antibody for 7 days. TRAP-positive cells with > 3 nuclei were counted as osteoclasts. The results shown are the mean ± SD of three independent experiments. $*p<0.05$; $**p<0.01$.

Figure 6 is a series of photographs of gels, which show CCR1 chemokine receptor expression in RANKL-induced osteoclasts. Osteoclasts were induced by RANKL from RAW264.7 and bone marrow cells. mRNA specific for CCR1 was amplified by semi-quantitative RT-PCR for CCR1 and GAPDH as a control. PCR was performed under conditions determined to be in the liner range of product formation.

Figure 7 is a line graph of MIP-1γ enhancement of osteoclast survival. RAW264.7 cells were stimulated with RANKL (10 ng/ml) for 5 days, extensively washed, and subsequently stimulated with RANKL (10 ng/ml), MIP-1γ (2 ng/ml) or medium as a control. Neutralizing anti-MIP-1γ antibody (5 μg/ml) or control IgG_1 antibody (5 μg/ml) were simultaneously added with RANKL to determine the role of MIP-1γin RANKL-stimulated osteoclast survival. TRAP-

positive cells with >3 nuclei were counted as osteoclasts. The results shown are the mean ± SD of three independent experiments. *$p<0.05$ RANKL + control antibody vs RANKL + anti-MIP-1γ.

Figures 8A-8B are a series of photographs (A) and an autoradiograph (B). (A) Shows the anti-apoptotic effect of MIP-1γ on mature osteoclasts. RANKL induced osteoclasts were washed and re-cultured with (1) medium, (2) RANKL, (3) MIP-1γ (4) RANKL + anti-MIP-1γ antibody (5 μg/ml), or (5) RANKL + control IgG_1 antibody (5 μg/ml) for 24 hr. Cell staining with Rhodamine 110 (green) indicates caspase-3 activity; TOTO-3 (blue) counterstains nuclei. Arrowhead: osteoclast. Magnification: 100x. (B) Effect of MIP-1γ on NF-κB activation in osteoclasts. Osteoclasts were generated by RANKL stimulation of RAW264.7 cells for 5 days, washed, and re-stimulated with RANKL (10 ng/ml) (lane 4), MIP-1γ (10 ng/ml)(lane 5) or medium (lane 3) as a control for 12 hr. Nuclear extracts were assessed for NF-κB activity by EMSA. Positive control: day 5 RANKL-stimulated osteoclasts (lane 2); negative control; no nuclear extract (lane 1).

Figures 9A-9B are a series of photomicrographs (A) and a bar graph (B) showing that MIP-1γ enhances the activation of pre-formed osteoclasts. RAW264.7-derived osteoclasts recovered from collagen gel cultures, and were plated on Osteologic Multitest Slide™ to assess resorptive activity without/with RANKL (10 ng/ml) or MIP-1γ(2, 5 and 10 ng/ml). (A) Photomicrograph of resorption areas visualized by light microscopy (10X). (B) The size of resorbed areas was quantified using NIH Image™. Data represent the mean ± SD of triplicate cultures.

Figures 10A-10C are bar graphs (A-C) and an autoradiograph (D) showing RANKL-induced transcriptional activation in osteoclast differentiation. **A,** Transcriptional screening analysis performed on macrophage-like RAW264.7 cells at 30min and 3 days after RANKL stimulation showed a time-dependant activation of Brn3. The screening system was evaluated by the activation profiles of the transcription factors NF-κB, AP-1 and NFATc known to be activated in osteoclastogenesis. Activation ratios above 1.3 were considered as an increase in transcriptional activity compared to unstimulated cells. **B,** Time course of RANKL induced Brn3/ DNA-binding activity; combined results of three separate experiments. *$p<0.01$, compared to non-stimulated cells. **C,** Supershift experiments showed the presence of Brn3a and Brn3b in

the Brn3/DNA binding complex. A specific antibody was not available for Brn3c. Combined results of three separate experiments. *$p<0.05$, compared to day 4 nuclear extract.

Figures 11A-11D are a series of photographs (A), a line graph (B), and bar graphs (C and D) showing the induction of Brn3 gene and protein expression during osteoclast differentiation. **A**, Immunofluorescence staining illustrates nuclear and cytoplasmic localization of Brn3a, Brn3b and Brn3c in RAW264.7-derived osteoclasts after 4 days in culture. **B**, Expression of *brn3a, brn3b* and *brn3c* mRNA increases in response to RANKL stimulation of RAW264.7 cells. *$p<0.05$ vs. baseline. **C**, Brn3a and Brn3b Western blot analysis revealed an increase in protein levels after stimulation with RANKL. Combined results of three separate experiments. *$p<0.05$ vs. baseline.

Figures 12A-12E are a series of photographs (A), bar graphs (B and E), an autoradiograph (C), and a photograph of a gel (D) showing the functional inhibition of transcriptional activity by Brn3 decoy oligonucleotides. **A**, FITC labeled decoy and mutant oligonucleotides as a control were transfected into cells, and transfection efficiency was monitored by fluorescence microscopy (FITC). BF: brightfield. **B**, Decoy oligonucleotides almost completely abolished RANKL-induced Brn3 binding activity in nuclear extracts, compared to mutant transfected control cells. **C**, RANKL stimulated expression of the Brn3 target gene synaptotagmin 1 (Syt 1) was inhibited by functional blockade of Brn3. *$p<0.05$ vs. mutant transfected cells.

Figures 13A-13C are a series of photographs (A), a line graph (B), and a bar graph (C) showing the reduction of giant cell formation by functional inhibition of Brn3. **A**, Brightfield (BF) and fluorescence microscopy (FITC) of RANKL-induced RAW264.7 cells transfected with FITC-labeled oligonucleotides. As indicated by arrows, FITC-labeled Brn3 mutant oligonucleotides transfected into cell nuclei did not interfere with giant cell formation. In the Brn3 decoy oligonucleotide culture, multinucleated giant cells appear almost non-transfected compared to cells that integrated Brn3 decoy oligonucleotides and therefore remained smaller and less differentiated. **B,** Functional inhibition of Brn3 by using decoy oligonucleotides resulted in a 40% reduction of giant cell formation assessed by total osteoclast surface area. *$p<0.05$ vs. mutant transfected cells. **C,** Effect of functional inhibition of Brn3 on multinuclearity of osteoclasts. *$p<0.05$ vs. mutant transfected cells.

Figures 14A-14C are bar graphs with accompanying photographs showing that the functional inhibition of Brn3 reduces osteoclast resorptive activity. **A**, RANKL-induced osteoclastic resorption activity is diminished in differentiated RAW264.7 cells transfected with decoy oligonucleotides. Resorption was assessed on submicron calcium phosphate slides. *$p<0.01$ vs. mutant transfected cells. **B**, RANKL-induced osteoclasts derived from normal bone marrow cells. **C,** RANKL-induced osteoclasts derived from RAW264.7 cells exhibited greater than 80% reduction in pit formation on bovine bone slides by functional inhibition of Brn3; scanning electron microscopy. *$p<0.05$ vs. mutant transfected cells.

Figures 15A-15H are a series of photographs (A) and bar graphs (B-H) showing that *Brn3b* gene deletion results in increased bone mass and altered bone micro- architecture *in vivo*. **A**, Representative micro-CT sections of femurs from wild type (WT) and homozygous Brn3b knockout mice (HO). **B**, Bone mineral density of wild type, heterozygous (HE) and homozygous knockouts was determined by densitometry. **C-H:** Micro-CT derived parameters of femurs in WT, HE and HO mice. **C,** apparent bone volume; **D**, cortical percent bone volume; **E**, cortical thickness; **F**, cortical percent marrow volume; **G,** trabecular separation; **H**, trabecular number. **$p<0.01$, *$p<0.05$.

DETAILED DESCRIPTION OF THE INVENTION

MIP-1 Gamma Promotes RANKL-Induced Osteoclast Formation and Survival

Osteoclastic bone resorption consists of multiple steps, including the differentiation of osteoclast precursors, the fusion of mononuclear cells to form mature multinucleated osteoclasts, activation to resorb bone and finally the survival of activated osteoclasts. RANKL (Receptor Activator of NF-κB Ligand), also known as TRANCE or OPGL, is a member of the TNF family and is one of key molecules that regulates both osteoclastogenesis and bone resorption. RANKL expression by osteoblasts as well as by activated T cells has been shown to regulate these processes. However, the participation of additional factors, including autocrine factors induced by RANKL stimulation, is less well characterized.

Chemokines play an important role in immune and inflammatory responses by inducing the migration and adhesion of leukocytes. Several chemokines regulate the migration and differentiation of OC, including MIP-1α and IL-8. However, the cellular source(s) of these chemokines, and their role in the overall regulation of bone mass remains unclear.

Macrophage inflammatory protein-1 gamma (MIP-1γ) is a CC chemokine family member. MIP-1γ induces the chemotaxis of $CD4^+$ and $CD8^+$ T cells and monocytes *in vitro*, and shows potent suppressive activity on the colony formation of murine bone marrow myeloid progenitor cells. MIP-1γ mRNA is widely expressed in most tissues of normal mice except brain.

Using gene microarrays, we found that MIP-1γ mRNA expression was strongly upregulated in RANKL-induced OC. MIP-1γ is involved in RANKL-induced OC formation, survival, and activation in bone resorption, likely via an autocrine pathway.

Mice and reagents

Three to five week-old BALB/c mice were obtained from the Jackson Laboratory, Bar Harbor, ME. Recombinant mouse RANKL (RANKL) and recombinant mouse M-CSF (M-CSF) were purchased from PeproTech Inc (Rocky Hill, NJ). Recombinant mouse MIP-1γ, anti-mouse MIP-1γ antibody and control IgG_1 antibody (IgG_1 antibody) were obtained from R&D Systems (Minneapolis, MN).

Cell culture and differentiation of RAW264.7 and bone marrow cells.

RAW264.7, a mouse macrophage/monocyte cell line, was purchased from ATCC (TIB-71). Cells were cultured in Dulbecco's modification of Eagle's medium (DMEM; JRE Biosciences, Lenexa, KS) supplemented with 10% FBS (Gibco, Grand Island, NY), 1.5 g/l sodium bicarbonate and penicillin/streptomycin (Gibco). To generate osteoclasts, RAW 264.7 cells were plated in 24-well plates at a density of 1×10^4 cells/well. Cells were stimulated with 10 ng/ml of recombinant mouse RANKL for 5 days. Mouse bone marrow (BM) cells were collected from femora and tibiae. Three to five week-old female mice were killed by cervical dislocation under light ether anesthesia. Femora and tibiae were dissected, BM cells were flushed out and cultured in α-MEM (Cambrex, Walkersville, MD) supplemented with 10% FBS, 2.0 g/l sodium bicarbonate and penicillin/streptomycin. BM cells were seeded into 24-well plates at a density of 2×10^6 cells/well in medium supplemented with 20 ng/ml of RANKL and 50 ng/ml of recombinant mouse M-CSF for 7 days. All cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air, with changes of medium every other day. Osteoclast numbers were evaluated by counting tartrate-resistant acid phosphatase (TRAP)-positive giant

cells. At culture termination, cells were washed with PBS and fixed in 10% formalin for 5 min followed by ethanol/acetone (1:1) for 1 min. Osteoclasts were stained for TRAP in the presence of 0.05 M sodium tartrate (Sigma-Aldrich, St. Louis, MO), napthol AS-MX phosphate (Sigma-Aldrich) as substrate, and fast red LB salt (Sigma-Aldrich). TRAP-positive multinuclear cells (3 or more nuclei/cell) were counted under light microscopy.

Gene array analysis

Two array systems were used to detect differences in gene expression between undifferentiated precursor cells and osteoclasts: the Atlas™ Mouse 1.2 array (Clontech Laboratories, Inc. Palo Alto, CA) and the MG-U73 chip (Affymetrix Santa Clara, CA). To enrich for differentiated OC, RANKL-stimulated cultures were gently trypsinized for 1 min to remove other less adherent cells. This treatment generated a population of >80% OC.

For analysis of gene expression in OC, total RNA was isolated from both undifferentiated cells and purified OC using TRIzol reagent (Invitrogen Co., Carlsbad, California). Total RNA was subsequently treated with DNase I, (Ambion, Austin, TX) to remove contaminating genomic DNA and quantified by spectrophotometry. The Atlas™ array, was hybridized to a radioactively labeled mixed cDNA probe obtained by reverse transcription of 4 μg of total RNA, according to the manufacturer's instructions. After hybridization, the arrays were washed to remove unbound probe and exposed to X-ray film. The level of gene expression was analyzed and normalized using NIH Image™ software. The MG-U73 chip was hybridized to a biotinylated mixed cDNA probe, washed and stained according to the standard Affymetrix GeneChip protocol. The level of gene expression was analyzed and normalized using statistical algorithms provided by Affymetrix.

Modulation of RANKL-induced osteoclastogenesis

MIP-1γ (0.1, 0.5, 2.0 ng/ml) was added to cultures of RAW264.7 and BM cells to examine its effect on RANKL-induced osteoclast differentiation. To eliminate possible effects of contaminating LPS, 1 μg/ml of polymyxin B (Sigma-Aldrich) was simultaneously added to some cultures. To assess the effect of endogenous MIP-1γ, anti-MIP-1γ antibody or IgG_1 control antibody (0.5, 5.0 μg/ml) was added to cultures of RAW264.7 and BM cells stimulated with RANKL.

Measurement of chemokines

Chemokines were measured in culture supernatants and cell lysates of RAW264.7 and BM cells. After supernatants were collected, cells were washed with PBS and lysed in 500 μl of protein extraction buffer (0.5% Triton-X100, 50 mM Tris-HCl, 0.3 M NaCl and 5 mM EDTA). MIP-1γ, MIP-1α and RANTES levels were determined using commercially available ELISA kits (R&D Systems).

Semi-quantitative RT-PCR

Semi-quantitative RT-PCR was used to examine CCR1 receptor gene expression in undifferentiated cells and purified osteoclasts. For this, 1 μg total RNA was reverse transcribed using Superscript II (Invitrogen) and random primers according to the manufacturer's instructions. cDNA was subjected to PCR amplification with *Taq* polymerase (Qiagen, Valencia, CA) using specific mouse CCR1 primers: sense: 5'-GTGTTCATCATTGGAGTGGTGG-3' (SEQ ID NO:1), antisense: 5'-GGTTGAACAGGTAGATGCTGGTC-3' (SEQ ID NO:2).

Evaluation of osteoclast survival and activity

For cell survival analysis, OC's were generated from RAW264.7 cells for 5 days. Adherent OC's were washed extensively with PBS to completely remove RANKL. Cells were subsequently cultured without or with 10 ng/ml of RANKL in the presence/absence of 2.0 ng/ml of MIP-1γ. Neutralizing anti-MIP-1γ antibody (5 μg/ml) or control IgG_1 antibody (5 μg/ml) was simultaneously added with RANKL to determine the role of MIP-1γ in apoptosis of RANKL-induced osteoclasts. After 24, 48, and 72 hr, survival was determined by counting adherent TRAP-positive osteoclasts.

To determine the effect of MIP-1γ on bone resorbing activity, OC's were generated in three-dimensional collagen gels (Chemicon, Temecula, CA). Sixty mm dishes were covered with a collagen gel solution prepared according to the manufacturer's instructions. RAW264.7 cells were seeded onto the gels and cultured with 10 ng/ml of RANKL for 5 days. Cells were removed following digestion of gels with 1000 U/ml collagenase (Sigma-Aldrich) at 37°C for 30 min. Aliquots of the harvested cell suspension were seeded onto sub-micron calcium phosphate

films (Osteologic™; BD Biosciences, Bedford, MA) in 250 µl of medium. Cells were unstimulated (control), or stimulated with 10 µg of RANKL or MIP-1γ (2.0, 5.0, 10 ng/ml). After 24 hr, the cells were removed with 5 % sodium hypochlorite and resorbed areas were visualized using light microscopy. The size of resorbed areas was quantified using NIH Image™.

Preparation of Nuclear Extracts

Osteoclasts were generated by RANKL stimulation of RAW264.7 cells for 5 days, and were enriched by a brief trypsinization which removed most mononuclear cells. Osteoclasts were washed twice with PBS, pH 7.4, followed by suspension in 800 µl ice-cold lysis buffer (mmol/l: HEPES 10; KCL 10; EDTA 0.1; EGTA 0.1; DTT 1.0; PMSF 1.0; aprotinin 10 µg/ml, pepstatin 10 µg/ml, leupeptin 10 µg/ml). The collected samples were incubated on ice for 30 min, vortexed for 30 sec after addition of 50 µl 10% Nonidet-P40, and centrifuged for 10 min at 4°C at 5,500 xg. The nuclei-containing pellets were suspended in ice-cold buffer (mmol/l: HEPES 20; NaCl 400; EDTA 1.0; EGTA 1.0; DTT 1.0; PMSF 1.0; aprotinin 10 µg/ml, pepstatin 10 µg/ml; leupeptin 10 µg/ml), incubated on ice for 2h with frequent mixing, and centrifuged for 10min at 4°C at 14,000 xg. The supernatants were collected as nuclear extract and stored at –70°C. The total protein concentration was determined using a protein assay kit (Pierce, Rockford, IL).

Electrophoretic Mobility Shift Assays (EMSA)

NF-kB binding studies were performed using double stranded oligonucleotides containing an NF-kB consensus binding site. The oligonucleotides were end-labeled with ^{32}[P]-ATP using T4 polynucleotide kinase (Promega, Madison, WI) and incubated with the nuclear extract for 20 min at room temperature. The samples were loaded on a 4% non-denaturing polyacrylamide gel. After electrophoresis, the gel was dried and exposed to Kodak film.

Apoptosis Assays

Caspase-3 activity and degradation of DNA were examined for detection of apoptosis of OC's. OC's were obtained by RANKL stimulation of RAW 264.7 cells for 5 days, extensively washed, and re-stimulated with MIP-1γ or RANKL in the presence/absence of anti-MIP-

1γantibody. After 24 hr, cells were fixed in 4% paraformaldehyde, and were labeled with the caspase-3 substrate Rhodamine 110 (100 μM) and 2.4 nM of TOTO-3 for nuclear staining (Molecular Probes, Eugene, OR) at 37°C for 30 min. Cells were washed and viewed using a fluorescence microscope. Apoptotic cells exhibited bright green fluorescence. For DNA fragmentation determinations, OC's were washed with PBS, and lysed in a buffer consisting of 5 mM Tris-HCl (pH 8.0), 20 mM EDTA, 0.5% (v/v) Triton X-100 and 0.1% SDS. High-molecular mass DNA was removed by centrifugation at 14000 x g for 30 min. The supernatants were sequentially extracted with equal volumes of phenol/chloroform/isoamyl alcohol (25:24:1), and the soluble DNA was precipitated with 1 ml ethanol in the presence of 0.3 M sodium acetate. The DNA was resuspended in TE buffer and treated with DNase-free RNase (0.1 μg/μl) (Roche Diagnostics Corporation, Indianapolis, IN) for 1 hr at 30°C. DNA fragmentation was analyzed by 1.5% agarose-gel electrophoresis.

Statistical analysis

In all studies, differences between groups were analyzed using Student's *t* test with the Bonferroni correction for multiple comparisons.

Induction of MIP-1γ expression by RANKL during osteoclastogenesis

Osteoclast formation was induced by RANKL stimulation of RAW 264.7 macrophages for 5 days, or by stimulation of normal mouse bone marrow cells for 7 days with M-CSF and RANKL. RANKL-stimulated RAW264.7 and bone marrow cells differentiated into TRAP positive osteoclasts that expressed high levels of the osteoclast markers TRAP, cathepsin K and the proton pump subunit ATP6I, and produced resorption pits on bone slices and calcium phosphate-coated slides.

Two gene array systems (Atlas™ and Affychip™) were used to study gene expression following RANKL induction of osteoclast formation. Total RNA was extracted from RANKL-induced osteoclasts, and was used as a template to generate mixed cDNA probes. In the Atlas system (1100 genes), a highly significant up-regulation of mRNA was obsweved for the chemokine MIP-1γ in RANKL-stimulated osteoclasts derived from RAW264.7 cells, compared to un-stimulated precursor cells (Table 1). This result was confirmed and extended using the

Affymetrix system (32,000 genes) and mRNA derived from osteoclasts induced from normal bone marrow as well as RAW264.7 cells. The induction of MIP-1γ was more significant than any other chemokine or cytokine genes represented on these arrays.

Table 1: Array analysis of MIP-1γ gene expression in osteoclasts

Atlas™	**RAW264.7**	
Differentiated osteoclasts/undifferentiated cells	5.54*	
MG-U73 chips	**RAW264.7**	**Bone Marrow**
Differentiated osteoclasts vs undifferentiated cells	<0.000001**	<0.000001**

*Ratio of intensity of gene expression, normalized to housekeeping genes.
**p value, Wilcoxon's signed rank test.

Expression of C-C chemokines in developing osteoclasts

MIP-1γ binds to the CCR1 receptor, which is also activated by related C-C chemokines MIP-1α and RANTES. The production of MIP-1γ, MIP-1α, and RANTES proteins was examined during the process of RANKL-induced osteoclastogenesis from RAW264.7 cells. As shown in Figure 3A, RANKL strongly stimulated the production of MIP-1γ in both cell lysates and culture supernatants over the 5-day culture period. MIP-1α was also induced by RANKL at about 10-fold lower levels. The levels of MIP-1α in supernatants peaked by day 2, although cell-associated levels continued to increase to day 5. RANTES was also weakly induced by RANKL, at levels about 1-2% of those of MIP-1γ at any time point (maximum: 50 pg/ml).

MIP-1γ was similarly induced following RANKL stimulation of normal bone marrow cells (Figure 3B). However, compared to RAW264.7 cells, RANTES was expressed initially at higher levels, but expression declined with increasing times after culture induction, and MIP-1α was nearly undetectable. MIP-1 γ is the predominant C-C chemokine produced by RANKL-stimulated precursor cells during osteoclastogenesis.

CCR1 receptor expression by osteoclasts

RAW 264.7 and bone marrow cells were examined for the expression of CCR1 receptor mRNA upon RANKL stimulation. As shown in Figure 3, CCR1 mRNA was undetectable in unstimulated RAW 264.7 cells, whereas it was present at low levels in unstimulated bone

marrow cells. RANKL stimulation strongly induced CCR1 mRNA in RAW 264.7 and, to a lesser extent, in bone marrow cells. Thus, RANKL induces both MIP-1γ and its receptor during osteoclast differentiation, indicating the operation of an autocrine pathway.

Modulation of osteoclastogenesis MIP-1γ

The role of endogenously produced MIP-1γ was examined in RANKL-induced osteoclastogenesis in RAW 264.7 and normal bone marrow cells. A neutralizing anti-MIP-1γ antibody was added to cultures beginning on day 0, and was replenished periodically throughout the osteoclast induction period. As seen in Figure 5, in both cell systems the addition of anti-MIP-1γ antibody resulted in a decreased number of TRAP positive osteoclasts, relative to control IgG1 antibody. The reduction was approximately 60%, in cultures treated with 5 μg/ml of anti-MIP-1γ antibody, and 45% in cultures treated with 0.5 μg/ml antibody. These findings were replicated in two additional experiments.

The effect of adding exogenous recombinant MIP-1γ on osteoclast formation was also determined. As seen in Figure 6, TRAP positive osteoclasts were not induced by MIP-1γ alone, nor did exogenous MIP-1γ have a synergistic effect with RANKL on osteoclastogenesis in either cell system. These results are consistent with the high level of endogenous MIP-1γ production. Endogenous MIP-1γ increases RANKL-induced osteoclast formation, but has no independent ability to induce osteoclastogenesis.

Effect of MIP-1γ on osteoclast survival

Mature osteoclasts rapidly undergo apoptosis in the absence of bone resorptive stimuli such as RANKL, LPS or IL-1α. RANKL was removed from cultures of differentiated osteoclasts by extensive washing of cells on day 5. Cells were then recultured for an additional 24 to 72 hours in the presence or absence of MIP-1γ or RANKL as a positive control. As shown in Figure 7, the number of osteoclasts was reduced by 90% after 24 hours in the absence of a stimulating agent (medium alone). RANKL re-stimulation promoted osteoclast survival, as indicated by a 30% reduction in viable TRAP positive cells after 24 hours. The addition of MIP-1γ alone also prevented cell death, albeit somewhat less effectively than RANKL itself. As indicated (Figure 7), the pro-survival activity of RANKL was reduced by approximately 60% in

the presence of anti-MIP-1γ antibody whereas an isotype-matched control IgG1 antibody had no effect.

That the loss of osteoclasts was the result of apoptosis rather than necrosis was demonstrated using a fluorescence-based assay for the specific activity of caspase 3 (Figure 8A). Osteoclasts cultured in the presence of RANKL exhibited minimal apoptosis (panel 2), compared to cells from which RANKL was removed (panel 1). Cells cultured with MIP-1γ alone were partially protected from apoptosis (panel 3). The pro-survival inhibiting effect of RANKL was demonstrated to be partially dependent on its ability to induce MIP-1γ, as shown by anti-MIP-1γ antibody blockade of the protective effect of RANKL (panel 4 vs 5). These results were further confirmed in parallel by DNA fragmentation studies.

Given that many of the factors that promote osteoclast survival, including RANKL, act by stimulating NF-κB, the effect of MIP-1γ on NF-κB transcription factor was examined. As shown in Figure 8B, following extensive washing of mature osteoclasts, RANKL restimulation strongly induced NF-κB DNA binding activity in mature osteoclasts, as assessed by electrophoretic mobility shift assay. MIP-1γ by itself also stimulated NF-κB, but less strongly than RANKL, which correlated with the level of its pro-survival activity (Figure 8A). Thus, MIP-1γ promotes the survival of mature osteoclasts by preventing apoptosis.

Effect of MIP-1γ on bone resorbing activity of mature osteoclasts

RANKL-induced RAW264.7 cells were cultured in three-dimensional collagen gels, isolated by enzymatic digestion, and replated onto sub-micron calcium phosphate films for an additional 24 hours in the presence/absence of MIP-1γ or RANKL as a positive control. As shown in Figure 9, osteoclasts were stimulated to form numerous resorption pits in the presence of added RANKL, but not in its absence. The addition of MIP-1γ alone also resulted in a marked stimulation of resorption, to a level similar to that seen with RANKL stimulated cells. Therefore, MIP-1γ stimulates the activation as well as the survival of mature osteoclasts.

Extensive cross talk occurs between the immune and skeletal systems. In particular, the differentiation and activity of osteoclasts, and hence bone mass, can be modulated by cytokines/chemokines, many of which derive from immune cells. Gene arrays were used herein

to identify mediators that were up-regulated following RANKL stimulation of osteoclast precursor cells. MIP-1γ was most strongly up-regulated in osteoclasts derived from RANKL-stimulated monocyte/macrophages. Expression of CCR1, the high affinity receptor for MIP-1γ, was also increased following RANKL stimulation. Inhibition of MIP-1γ expression or activity resulted in decreased osteoclast formation and reduced resorptive activity. Furthermore, MIP-1γ promoted osteoclast survival and prevented apoptosis, and was responsible for a major proportion of the pro-survival activity of RANKL itself. MIP-1γ regulates osteoclastic bone resorption, via effects on cell differentiation, survival and activation.

MIP-1γ is a C-C chemokine with a predicted length of 100 amino acids, which is identical with CCF18. MIP-1γ is constitutively expressed by a wide variety of tissues, and exclusively binds to the CCR1 receptor on mouse neutrophils *in vitro*. MIP-1γ expression is increased in rat bone marrow cells stimulated with RANKL.

Two other C-C chemokines, MIP-1α and RANTES, also bind to CCR1, and are therefore involved in modulating osteoclast development and function. However, MIP-1γ iss by far the predominant chemokine protein produced by RANKL-stimulated RAW264.7 and bone marrow cultures, compared to relatively minor amounts of MIP-1α and RANTES. MIP-1α and IL-8 inhibit the resorption of isolated rat osteoclasts, and these chemokines participate in osteoclast migration, but not resorption and survival. Conversely, increased levels of MIP-1α, which is produced and secreted by osteoblasts, correlates with an increase in the number of osteoclasts in a porcine bone marrow culture system. MIP-1α also stimulates osteoclast formation in human marrow cultures and also enhances formation of osteoclasts induced by PTHrP and RANKL. The RANTES gene is upregulated in RANKL-induced osteoclasts derived from mouse bone marrow cells. Neutralization of MIP-1γ reduced osteoclast formation by approximately 60%. MIP-1α and RANTES also participate in RANKL-induced osteoclastogenesis following interaction with CCR1, albeit with a more modest effect. Alternatively, residual MIP-1γ independent RANKL-induced osteoclast formation may proceed via pathways independent of these chemokines.

Osteoclasts rapidly undergo apoptosis unless stimulated by exogenous mediators, which include M-CSF, RANKL, IL-1, FGF2, or LPS. With the exception of M-CSF and FGF2, these survival-promoting stimuli act by inducing NF-κB, which is an anti-apoptogen. FGF-2, which

directly stimulates activation and survival of mature osteoclasts, mediates its effects through p42/p44 MAP kinase. MIP-1γ, like RANKL, stimulated NF-κB activity, and the pro-survival activity of RANKL was partially dependent on its ability to induce MIP-1γ. MIP-1γ also stimulated the activation of mature osteoclasts. Thus, in contrast to most survival factors that act in a paracine manner, osteoclasts protect themselves from apoptosis through production of MIP-1γ as an autocrine survival factor.

MIP-1γ is an important factor in the bone microenvironment that regulates osteoclastic bone resorption, and plays a role in both normal bone turnover and in osteolytic diseases.

MIP-1γ

MIP-1γ is also referred to herein as Scya9 (small inducible cytokine A9), which corresponds to OC # 3 (Figure 1A).

MIP-1gamma is closely related to MIP-1alpha, MIP-1beta, and C10. It shares a high-affinity receptor with MIP-1alpha and activates calcium release in neutrophils. Moreover, MIP-1alpha and MIP-1gamma are cross-desensitizing, suggesting that they utilize a common signalling pathway. Like MIP-1alpha, MIP-1gamma is pyrogenic in mice. However, distinct from other chemokines, MIP-1gamma is constitutively expressed by many tissues in vivo and circulates at relatively high concentrations in the plasma of normal mice. Presumably, it occupies a large fraction of the receptors available for CC chemokines in the plasma compartment.

U49513

> >gi|1234834|gb|U49513.1|MMU49513 Mouse macrophage inflammatory protein-1gamma mRNA, complete cds

> GGGCCAGCTGGGTCTGCCCACTAAGAAGATGAAGCCTTTTCATACTGCCCTCTCCTTCCTCATTCTTACA
> ACTGCTCTTGGAATCTGGGCCCAGATCACACATGCAACAGAGACAAAAGAAGTCCAGAGCAGTCTGAAGG
> CACAGCAAGGGCTTGAAATTGAAATGTTTCACATGGGCTTTCAAGACTCTTCAGATTGCTGCCTGTCCTA
> TAACTCACGGATTCAGTGTTCAAGATTTATAGGTTATTTTCCCACCAGTGGTGGGTGTACCAGGCCGGGC
> ATCATCTTTATCAGCAAGAGGGGGTTCCAGGTCTGTGCCAACCCCAGTGATCGGAGAGTTCAGAGATGCA
> TTGAAAGATTGGAGAAAAACTCACAACCACGGACCTACAAACAATAACATTTGCTTTAGAGAAGGGTGTG
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> CACATAATATTTGATTGAGATAAATGGGCAAGGTCTGGAGAGATGGCTTGGTGGTTAAGAGCACCTGCTG
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> AGAAAACTGATGCCCTATTTTGCCCCTTTAGTTAGTAGTATTTACAGTATTCTTTATAAATTCACCTTGA
> CATGACCATCTTGAGCTACAGCCATCCTAACTGCCTCAGAATCACTCAAGTTCTTCCACTCGGTTTCCCA
> GCGGATTTTAAGTGGATAAACTGTGAGAGTGGTCTGTGGGACTTTGGAATGTGTCTGGTTCTGATAGTCA

> CTTATGGCAACCCAGGTACATTCAACTAGGATGAAATAAATTCTGCCTTAGCCCAGTAGTATGTCTGTGT
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The ORF is nucleotides 29-397.

Mouse macrophage inflammatory protein-1gamma

protein_id=AAB02198.1/db_xref="GI:1234835"

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GGCTRPGIIFISKRGFQVCANPSDRRVQRCIERLEKNSQPRTYKQ (SEQ ID NO:4)

CCR1

This gene encodes a member of the beta chemokine receptor family, which is predicted to be a seven transmembrane protein similar to G protein-coupled receptors. The ligands of this receptor include macrophage inflammatory protein 1 alpha (MIP-1 alpha), regulated on activation normal T expressed and secreted protein (RANTES), monocyte chemoattractant protein 3 (MCP-3), and myeloid progenitor inhibitory factor-1 (MPIF-1). Chemokines and their receptors mediated signal transduction are critical for the recruitment of effector immune cells to the site of inflammation. Knockout studies of the mouse homolog suggested the roles of this gene in host protection from inflammatory response, and susceptibility to virus and parasite. This gene and other chemokine receptor genes, including CCR2, CCRL2, CCR3, CCR5 and CCXCR1, are found to form a gene cluster on chromosome 3p.

Homo sapiens chemokine (C-C motif) receptor 1 (CCR1), mRNA.

ACCESSION NM_001295

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The ORF is nucleotides 63-1130

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MIP-1α

Homo sapiens macrophage inflammatory protein-1-alpha/RANTES receptor mRNA, complete cds.

ACCESSION L10918

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ID NO:7)
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The ORF is nucleotides 63..1130.

macrophage inflammatory protein-1-alpha; protein_id=AAA36543.1; db_xref=GI:292417

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```

RANTES

RANTES (the acronym for "Regulated upon Activation, Normal T-cell Expressed and Secreted") is a small chemotactic protein that mediates many immunological, allergic, and inflammatory responses. RANTES is rapidly up-regulated in response to a variety of stimuli and, once secreted, it recruits monocytes, eosinophils, and basophils to the inflammation site. RANTES has been implicated in both acute and chronic phases of inflammation associated with pathological conditions such as asthma, autoimmune diseases, transplant reject, cancer and AIDS.

The human RANTES gene maps to chromosome 17q11. 2-q12 and spans approximately 7.1 kb. The mRNA transcribed from the RANTES gene is 1.2 kb long. It encodes a 10 kD protein, including a cleavable amino-terminal signal sequence of 23 amino acids. The secreted protein is 68 amino acids long with a predicted mass of approximately 8 kD.

Homo sapiens RANTES precursor, mRNA, complete cds.

ACCESSION AF043341

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The ORF is nucleotides 4-279.

protein_id=AAC03541.1; db_xref="GI:2905632"

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Terminal Differentiation of Osteoclasts is Regulated by the Brn3 Transcription Factors

The differentiation of pre-osteoclasts to mature osteoclasts following RANKL stimulation involves early activation of the transcription factors NF-κB and AP-1. Additional transcription factors are required for terminal differentiation and bone resorptive function (*e.g.* MITF)

Through screening arrays of transcription factor activity, we found that Brn3 family members are activated in osteoclast precursor cells in response to RANKL stimulation. Brn3 POU-domain transcription factors have been shown to be crucial in regulating differentiation and maturation of neuronal cells. There are three related proteins, Brn3a, Brn3b, and Brn3c (also known as Pou4f1, Pou4f2, Pou4f3) that are derived from distinct genes. Brn3 proteins share more than 95% identity within a bipartite DNA-binding POU domain, which consists of two highly conserved regions, connected by a variable linker of 14-26 amino acids. DNA binding site is ACTCATTAAT (SEQ ID NO:11), ACTCATTAAC (SEQ ID NO:12), GCTCATTAAT (SEQ ID NO:13), or GCTCATTAAC (SEQ ID NO:14) and similar sequences that are recognized by each of the Brn3 POU domains. Other Brn3 binding sites or target sequences include CACAGCTCATTAACGCGC (SEQ ID NO:44), CACTCCTCATTAACGCGC (SEQ ID NO:45), CACAGCTCATTAAGTCGC (SEQ ID NO:46), or CACGCATGCGTAATGCGC (SEQ ID NO:47). A general POU domain recognition site is GCATNNNTAAT (SEQ ID NO:48), where N represents any base pair. For the Brn3 protein class, high-affinity binding requires that N=3. Brn3 transcription factors can regulate specific target genes through interactions with these sites. The Brn3 class of proteins have been shown to bind with high

affinity to a recognition element derived from the rat corticotropin-releasing hormone (CRH) gene promoter, GCATAAATAAT (SEQ ID NO:49).

The human Brn3 POU domains are amino acids 270-418 in Brn3a, amino acids 257-405 in Brn3b, and amino acids 185-333 in Brn3c. The human Brn3 upstream homology domains are amino acids 44-70 in Brn3a, amino acids 98-123 in Brn3b, and amino acids 43-68 in Brn3c.

Brn3 family members are expressed mainly in neurons in the trigeminal and dorsal root ganglia, in retinal ganglion cells, in cochlear hair cells and in several brainstem nuclei, both during development and in adulthood, but have not previously been identified in bone.

The expression and activation of Brn3 family members during RANKL-induced osteoclast development and activation was examined. An increase in *brn3a, brn3b,* and *brn3c* expression was measured in response to RANKL stimulation of osteoclast precursor cells. In addition, DNA binding activity of Brn3a and Brn3b reached a maximum parallel to the appearance of first osteoclastic giant cells. Functional inhibition of Brn3 DNA-binding activity using decoy oligonucleotides containing a Brn3 consensus site resulted in an inhibition of pre-osteoclast fusion, and a reduction in the bone resorption activity of mature osteoclasts. Targeted deletion of *brn3b* in mice caused altered bone structure with increased bone mass. Thus, Brn3 transcription factors are important regulators of the terminal differentiation of osteoclasts that are targets for treatment of bone diseases.

Screening of Transcriptional Activation in Osteoclast Differentiation

Incubation of RAW 264.7 cells with RANKL, an art recognized model for osteoclast differentiation/function, for 5 days resulted in the formation of multinucleated osteoclast-like cells. As previously shown, these cells express the osteoclast-specific markers tartrate resistant acid phosphatase (TRAP), cathepsin K, matrix metalloproteinase-9 (MMP-9), the proton pump protein Atp6i, and form resorption pits on bone slices and sub-micron calcium phosphate films.

A transcriptional screening analysis was performed on RAW 264.7 cells after RANKL stimulation using the TranSignal Screen (Panomics, CA). The differential activation of 54 different transcription factor binding sites was explored over a time course of 3 days and compared to non-stimulated cells. This analysis revealed a specific time-dependent activity of Brn3 transcription factors, which increased significantly on day 3 (Figure 10A). In addition, NF-κB transcriptional activity increased after 30 min, and AP-1 and NFATc showed a delayed

increase of binding activity in response to RANKL stimulation, consistent with previous findings of other investigations.

To verify these results and identify the involved Brn3 transcription factors, EMSA analysis was performed using specific Brn3 consensus oligonucleotides. These experiments demonstrated a selective activation of the Brn3/DNA binding complex beginning on day 1, and peaking at day 4 after RANKL stimulation (Figure 10B). Supershift experiments revealed the presence of two related POU family members Brn3a and Brn3b in the Brn-3/DNA binding complexes (Figure 10C). In addition, immunohistochemical analysis by confocal microscopy demonstrated both nuclear and cytoplasmic localization of Brn3 proteins a, b, and c in RAW264.7 cells that were stimulated with RANKL for 4 days (Figure 11A).

Induction of Brn3 Gene and Protein Expression

Using semi-quantitative RT-PCR, a strong increase in expression of Brn3b and Brn3a mRNA was measured, along with a modest increase in Brn3c expression level that peaked on day 2 after RANKL stimulation (Figure 11B). Western blot analysis revealed a delayed increase in protein levels that peaked on day 3, with Brn3b showing higher expression than Brn3a (Figure 11C). Brn3 proteins are induced in response to RANKL stimulation and that Brn3b is the major POU4 family member expressed in developing osteoclasts.

Effect of Inhibition of Brn3 Transcriptional Activity by Decoy Oligonucleotides

Decoy oligonucleotides that contain a consensus binding site which is recognized by each of the Brn3 POU domain factors were used to inhibit the transcription activity of Brn3 proteins. The transfection efficiency of FITC-labeled decoy oligonucleotides was approximately 80% as assessed by fluorescence microscopy (Figure 12A). Transfected cells were extracted for their content of nuclear proteins and the residual Brn3 transcriptional activity was analyzed by EMSA. As seen in Figure 12B, Brn3 activity was reduced by approximately 70% compared to non-transfected cells or cells transfected with mutant oligonucleotides.

Decoy oligonucleotides in RANKL-stimulated RAW264.7 cells were used to examine the expression of synaptotagmin-1 (Syt1), a known Brn3 regulated gene. As shown in Figure 12C, Brn3 decoy oligonucleotides reduced Syt1 expression by 60% compared to control cells transfected with mutant Brn3 oligonucleotides. These data indicate that the transfection of RAW

264.7 cells with Brn3 decoy oligonucleotides results in a specific inhibition of the activity of these transcription factors *in vitro*.

Reduction of Osteoclast Fusion by Inhibition of Brn3 Transcriptional Activity

The effects of selective inhibition of Brn3 transcriptional activity on osteoclast formation was investigated. Cells were stimulated with RANKL, transfected on day 2, and monitored daily for RANKL-induced formation of osteoclastic giant cells from RAW264.7 cells. Transfection of Brn3 decoy oligonucleotides resulted in similar total numbers of TRAP+ osteoclasts. However, cell fusion was inhibited by up to 40% on day 5 compared to controls, as assessed by total osteoclast surface area (Figure 13A). FITC-labeled Brn3 mutant oligonucleotides were incorporated into cell nuclei without affecting cell fusion and differentiation. In contrast, those multinucleated cells that did form in the Brn3 decoy oligonucleotide cultures were consistently non-transfected, whereas cells that integrated Brn3 decoy oligonucleotides remained smaller and less differentiated (Figure 13A). Further analysis of multinucleated cells also demonstrated a significant reduction in the number of nuclei per cell due to inhibition of Brn3 by decoy oligonucleotides vs. cells transfected with Brn3 mutant oligonucleotides (Figure 13B). However, there was no reduction in the expression of either TRAP or cathepsin K by decoy oligonucleotides as assessed by Northern analysis. These results indicate that Brn3 proteins regulate very late events in osteoclast differentiation, in particular terminal cell fusion processes, but do not affect earlier gene expression.

Effect of Functional Inhibition of Brn3 on Osteoclast Bone Resorptive Activity

The impact of Brn3 on RANKL induced osteoclast resorptive activity was also analyzed. Differentiated RAW264.7 cells, seeded onto Osteologic™ slides and transfected with decoy oligonucleotides, exhibited 80% reduction of resorption activity in the decoy transfected cells compared to mutant oligonucleotide controls (Figure 14A). This result was confirmed using bovine bone slides. Functional inhibition of Brn3 by decoy oligonucleotides resulted in more than 80% reduction of the number of resorption pits compared to mutant control transfected cells (Figure 14C). Osteoclasts were generated by co-culture of bone marrow cells with calvarial cells in the presence of 10^{-8}M 1α, 25-$(OH)_2D_3$ for 7 days. After transfer of osteoclasts to Osteologic™ slides, cells were transfected with decoy or mutant oligonucleotides to block Brn3

activity. Similar to findings with RAW264.7 cells, a 41% reduction in osteoclastic resorption activity was seen following functional inhibition of Brn3 (Figure 14B). Thus, the Brn3 family are important factors in terminal stages of osteoclast differentiation and activation processes in response to RANKL stimulation.

Effect of Brn3 Gene Deletion on Bone Mass and Micro-architecture

Brn3b null mice are viable but exhibit severe damage in retinal cells, whereas Brn3a mice die shortly after birth. Therefore, the bone phenotype of Brn3b $^{+/+}$, $^{+/-}$ and $^{-/-}$ mice was determined by DEXA. Consistent with the *in vitro* findings, total bone mineral density was higher in the homozygous and heterozygous mice than in wildtype controls (Figure 15B). To further investigate the effect of *brn3b* deletion on trabecular and cortical bone, femurs were analyzed by micro-computed tomography (Figure 15A). As shown in Figure 15C, Brn3b null mice showed an increased bone volume fraction of the femur compared to both heterozygous and wildtype mice, confirming the DEXA findings. Analyses of cortical and trabecular bone in the midshaft region of the femur demonstrated higher cortical bone volume (Figure 15D), increased cortical thickness (Figure 15E), and a smaller bone marrow cavity (Figure 15F) in the Brn3b null mice compared to controls. In addition, trabeculae in Brn3b null mice showed less separation (Figure 15G) and a higher trabecular number. Therefore, Brn3b has an important role in maintenance of bone mass, likely via effects on osteoclast differentiation.

Induction and Activation of Brn3 Proteins by RANKL

The mature, multinucleated osteoclast derives from the monocyte/macrophage lineage in a differentiation process that ultimately involves the fusion of precursor cells to form multinucleated giant cells. As a mandatory inducing factor, RANKL activates osteoclast precursors to develop into bone resorbing cells. Differentiation results in profound morphological changes accompanied by a restricted gene expression pattern. The regulation of osteoclast differentiation is driven by a complex network of transcription factors, which includes NF-κB, AP-1, NFATc, E-box factor MITF and others.

To systematically analyze the transcriptional regulation of macrophage-like cells undergoing osteoclast differentiation an activated transcription factor array approach was used. One of the most prominently bound consensus binding sites was occupied by activated Brn3

POU domain factors. Analysis in more detail revealed a continually increasing activity of Brn3a and Brn3b peaking at day 4 *in vitro*, concurrent with detection of the first fused multinucleated osteoclasts. Since all the Brn3 proteins (a, b, c) have very similar DNA recognition and binding properties, a decoy oligonucleotide approach was chosen which was able to simultaneously inhibit the transcriptional activity of all three family members. The data clearly established that inhibition of Brn-3 proteins reduces osteoclast formation, fusion and activation *in vitro*. *In vivo*, Brn3b null mice had higher bone mass with increased cortical and trabecular bone.

Brn3 transcription factors were first described in neuronal differentiation, in which there is a clearly distinctive but overlapping pattern of *brn3a, brn3b, brn3c* gene expression, as well as in some non-neuronal cells such as in testis during germ cell development. The phenotypes exhibited by Brn3 null mice reflect the temporal expression patterns of the three POU4 transcription factors. In dorsal root and trigeminal ganglia, *brn3a* is the first of the Brn3 family members that is expressed. Brn3a gene deletion *in vivo* causes impairment of somatosensory and motor control in large part secondary to selective neuronal destruction in these regions. *Brn3b* is the first *brn3* gene to be detected in the developing retina followed by *brn3a* and *brn3c* expression. Brn3b null mice show severe defects in the retina and are missing about 70% of their retinal ganglion cells. In cochlear and vestibular hair cells of the inner ear, *brn3c* is first expressed, and *brn3c* absence leads to hair cells degeneration with a secondary loss of spiral and vestibular ganglion neurons.

Studies in chick ganglion cells indicate a functional redundancy of the Brn3 family members, since all showed a similar ability to promote cell differentiation and development when ectopically expressed in retinal progenitor cells. Brn3a and Brn3b could both function as transcriptional activators with no observed inhibitory effect of Brn3b on the transactivation activity of Brn3a, in contrast to earlier studies that described antagonistic effects of these factors. In addition, Brn3b/3c double knockout mice exhibit even more severe retinal ganglion cell defects, also indicative of a partial functional redundancy. In osteoclast precursor cells nearly equal *brn3a* and *brn3b* expression was seen in response to RANKL-stimulation, with less *brn3c* expression. However, a specific effect of *brn3b* deletion *in vivo* on bone structure and mass suggests a predominant role for Brn3b since its related family proteins Brn3a and Brn3c do not compensate its function. This is also consistent with findings that Brn3b can activate *brn3a* and Brn3a can regulate itself.

Brn3 is Essential for Terminal Osteoclast Differentiation and Bone Resorptive Activity

The functional effect of Brn3 transcription factors on osteoclast differentiation was investigated by transfecting decoy oligonucleotides into cells, which causes a suppression of Brn3 DNA binding activity. Functional inhibition of Brn3 factors resulted in a reduction of macrophage-derived multinucleated giant cell formation. The cells underwent morphological changes toward mature osteoclasts and expressed normal levels of TRAP and cathepsin K, but demonstrated a significant reduction in multinuclearity. This finding, along with the kinetics of Brn3 DNA binding activity, indicates an involvement of Brn3 proteins in late differentiation including terminal cell fusion processes. In addition, functional inhibition of Brn3 factors in differentiated macrophage-like RAW264.7 cells and in osteoclasts derived from co-cultured bone marrow cells decreased their bone resorptive activity dramatically.

Thus, it appears that Brn3 factors are downstream of NF-κB and AP-1, as well as another RANKL-induced intermediate-late transcription factor, MITF. In contrast to Brn3, MITF deficiency results in reduced expression of both TRAP and cathepsin K. Computer-based promoter analysis of the *brn3* family genes indicates the presence of several E-boxes (MITF/TFE3 responsive sites) in addition to multiple NF-κB binding sites. The phenotypic changes observed following inhibition of Brn3 proteins are consistent with effects on the cytoskeleton. Terminal differentiation and resorptive activity are reduced, the latter of which is highly dependent on the establishment of cell polarity with a change of internal cytoskeletal structures. Similarly, in retinal cells, studies of Brn3b null mice indicate that Brn3b is essential for controlling genes whose products are required for the organization of the cytoskeleton, normal cell polarity and axon outgrowth. Therefore, Brn3b is not required for the initial commitment of retinal ganglion cell fate, but without it the terminal differentiation and the formation of axons is disturbed. Similar effects of gene deletion on cell development were found in Brn3c null mice which show an initial generation and differentiation of hair cells but fail to fully develop to form stereociliar bundles. Forced expression of Brn3a *in vitro* led to outgrowth of mature neuronal processes under conditions when this would normally not occur.

Targeted Deletion of Brn3b Results in Impaired Bone Micro Architecture

Brn3b knockout mice exhibited increased total bone mineral density and bone volume fraction. Micro-architectural changes included thicker cortical bone, a higher number of trabeculae with decreased spacing, and a smaller bone marrow cavity. Since bone development and remodeling require fully functioning osteoclasts, Brn3b deletion results in an osteopetrosis-like syndrome, which, however, is milder than that induced by deletion of other RANKL-induced factors such as NF-κB and AP-1.

The activation of Brn3 transcription factors is enhanced in RANKL-induced osteoclast differentiation and the inhibition of Brn3 proteins results in a suppression of giant cell formation and a reduction in osteoclast resorptive activity. The Brn3 transcription factors are new targets for the modulation of bone mass, and the treatment of bone diseases caused by excessive bone resorption.

Cell Culture

Mouse macrophage-like RAW 264.7 cells (ATCC, Manassas, VA) were cultured in DMEM supplemented with 1.5g/l sodium bicarbonate and 10% fetal bovine serum (FBS, Invitrogen, CA). To induce osteoclast differentiation, recombinant mouse RANKL (R&D Systems, Minneapolis, MN) was added at a concentration of 10ng/ml. In some experiments, normal mouse bone marrow cells were obtained from the femurs of four week old BALB/c mice, and were cocultured with calvarial cells isolated from 1-2 day old newborn mice for 10 days in αMEM/10% FBS supplemented with 10^{-8} M 1α,25-$(OH)_2$D3 (Biomol, Plymouth Meeting, PA) to induce osteoclast formation.

Preparation of Nuclear Extracts

Cells were washed twice with PBS, pH 7.4, followed by suspension in 800μl ice-cold lysis buffer (mmol/l: HEPES 10; KCl 10; EDTA 0.1; EGTA 0.1; DTT 1.0; PMSF 1.0; aprotinin 10μg/ml, pepstatin 10μg/ml, leupeptin 10μg/ml). The collected samples were incubated on ice for 30min, vortexed for 30sec after addition of 50μl 10% Nonidet-P40, and centrifuged for 10min at 4°C. The nuclei-containing pellets were suspended in ice-cold buffer (mmol/l: HEPES 20; NaCl 400; EDTA 1.0; EGTA 1.0; DTT 1.0; PMSF 1.0; aprotinin 10μg/ml, pepstatin

10μg/ml; leupeptin 10μg/ml), incubated on ice for 2h with frequent mixing, and centrifuged for 10min at 4°C. The supernatants were collected as nuclear extract and stored at –70°C. The total protein concentration was determined using a protein assay kit (Pierce, Rockford, IL).

Screening of Transcriptional Activation

Nuclear extracts of cells at different times after RANKL stimulation (baseline, 30 min and 72 h) were incubated with biotin-labeled DNA-binding oligonucleotides (Panomics, Redwood City, CA). The protein/DNA complexes were separated from the free unbound probe by loading onto a 2% agarose gel in TBE buffer (M/l: TRIS1, boric acid 0.9, EDTA 0.01). After extraction of the protein/DNA complexes from the gel, the oligonucleotides were isolated following heating of the sample to 95°C for 3 min. The collected probes were hybridized overnight to a TranSignal Array membrane (Panomics) containing the consensus binding sequences for 54 different transcription factors. After conjugating with streptavidin-alkaline phosphatase (Perkin Elmer, Wilmington, DE), the bound probes were visualized on a Kodak film by using CDP-Star chemiluminescence reagent (Tropix, Bedford, MA).

Electrophoretic Mobility Shift Assays (EMSA)

Brn3 binding studies were performed using double stranded oligonucleotides containing a Brn3 consensus site (5`-CACAGCTCATTAACGCGC-3` (SEQ ID NO:16), 3`-GTGTCGAGTAATTGCGCG-5`(SEQ ID NO:17)) (Panomics, Redwood City, CA). The oligonucleotides were end-labeled with ^{32}-ATP using T4 polynucleotide kinase (Promega, Madison, WI) and incubated with nuclear extract for 20 min at room temperature. The samples were loaded on a 4% non-denaturing polyacrylamide gel. After electrophoresis, the gel was dried and exposed to Kodak film. Antibody supershift assays were carried out to confirm the identity of proteins in the Brn3 binding complex. Nuclear extracts were incubated with the appropriate antibody (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C followed by the addition of the labeled oligonucleotide probe.

RT-PCR

Total RNA was extracted using TRIZOL reagent (Invitrogen, Carlsbad, CA), reverse transcribed to cDNA with SuperScript II (Invitrogen, Carlsbad, CA), and used as a template for

the PCR reaction. The PCR was done using the following primers: brn3a sense: 5`GCCAACCTCAAG-ATCCCGGGCG`3 (SEQ ID NO:18); brn3a anti-sense: 5`CCAGTTTCTCGGCGATGGCGGC`3 (SEQ ID NO:19); brn3b sense: 5`CAC-GGTGGTGTCCACTCCGG`3 (SEQ ID NO:20); brn3b anti-sense: 5`CCGCGATCTTCTCCGAGGAG`3 (SEQ ID NO:21); brn3c sense: 5`GGCCATGAACGCCAAGCAGCCTTTCGGC`3 (SEQ ID NO:22); brn3c anti-sense: 5`GCGCCT-AGATGATGCGGGTGGATCTGCG`3 (SEQ ID NO:23); synaptotagmin 1(Syt 1) sense: 5`-GACCGCTTCTCC-AAGCACGAC-`3 (SEQ ID NO:24); Syt 1 anti-sense: 5`-CTGCGCCGGTGCTGTTGTAG-`3 (SEQ ID NO:25).

Western Blot Analysis

Cells were washed twice with PBS and lysed with buffer (mmol/l: Tris HCL 20; NaCl 150; EDTA 1; sodium orthovanadate 1; PMSF 0.5; 10μg/ml aprotinin; 1% Nonidet p40; 0.1% SDS). The samples were incubated on ice for 1.5 h, and centrifuged for 10 min at 4°C in an Eppendorf centrifuge. The supernatants were collected and stored at –20°C. The protein concentration was determined using a protein assay reagent (Pierce, Rockford, IL). The samples were mixed with loading buffer (Sigma, St. Louis, MO), heated at 95°C for 5 min, electrophoresed on a 12% SDS-polyacrylamide gel, and proteins were transferred onto a PVDF membrane. The proteins were detected with a primary anti-Brn3a or anti-Brn3b antibody (Santa Cruz Biotechnology, Santa Cruz, CA) using a Western blot kit (Invitrogen). Positive bands were visualized using the chemiluminescence reaction (Invitrogen) followed by exposure to photographic film.

Immunohistochemistry

Differentiated RAW264.7 cells were analyzed using a monoclonal antibody against Brn3a, and polyclonal antibodies against Brn3b (Santa Cruz Biotechnology, Santa Cruz, CA) and Brn3c (Covance Research Products, Berkeley, CA). The complementary secondary antibodies were conjugated with Alexa 488, Alexa 648 (Molecular Probes, Eugene, OR) and rhodamine (Santa Cruz Biotechnology, Santa Cruz, CA) respectively. Samples were analyzed on a Leica TCS SP2 confocal microscope.

Histological Analysis of Osteoclast Differentiation

Tartrate-resistant acid phosphatase (TRAP) was detected using a commercially available system (Sigma-Aldrich, St. Louis, Missouri). after RANKL stimulation, RAW264.7 or bone marrow cells were washed twice with PBS (pH 7.4), fixed, washed again, and incubated with TRAP staining solution for 30 min at 37°C. TRAP-positive cells containing three or more nuclei were counted as osteoclasts. Images of the osteoclasts were taken under a bright field microscope and normalized onto a standardized quadrangle. The measurement of total osteoclast area was done using the Scion program (NCBI).

Analysis of Osteoclast Resorptive Activity

RAW264.7 or bone marrow cells in coculture with calvarial cells were induced to differentiate for 4-5 days or 7-10 days, respectively, on a 3D Collagen Cell Culture System matrix following the manufacturer's instructions (Chemicon International Inc., Temecula, CA). Cells were removed by dissolving the matrix in 0.2% collagenase, and were replated on 16-well Osteologic Multitest Slides (BD Bioscience, Bedford, MA) or 100μm thick bovine bone slides. Cells were cultured for an additional 2 days in either DMEM/10%FBS with 10ng/ml RANKL (RAW264.7), or αMEM/FBS with 25ng/ml RANKL for osteoclasts derived from bone marrow. Resorption lacunae were photographed by bright field (Osteologic) or electron microscopy (bone slides) and analyzed using an image analyzing software (Scion Image NCBI).

Inhibition of Transcriptional Activation of Brn3-proteins

Double-stranded oligonucleotides were prepared from complementary single stranded phosphorothioate-bonded fluorescence-labeled oligonucleotides by melting at 95°C for 5 min followed by a cooling phase at room temperature. The sequences of the oligonucleotides were as follows: Brn3 sense: 5`-CACAGCTCATTAACGCGC-`3 (SEQ ID NO:26); Brn3 anti-sense: 5`-GCGCGTTAA-TGAGCTGTG-`3 (SEQ ID NO:27); Brn3 mutated sense: 5`-GCGCGTTGCTGAGCTCTG-`3' (SEQ ID NO:28); Brn3 mutated anti-sense: 5`-CAGAGCTCAGCAACGCGC-`3 (SEQ ID NO:29). All decoy oligonucleotides were transfected into the cells using Oligofectamine Reagent (Invitrogen, Carlsbad, CA) and efficiency of transfection was monitored by fluorescence microscopy.

Analysis of Bone Micro-architecture

The bone micro-architecture of mice carrying a targeted mutation of the *brn3b* gene was compared with heterozygous and wild type controls. Femurs were isolated and first analyzed by bone densitometry using dual-energy x-ray absorptiometry (DEXA, Piximus, Faxitron X-ray, Wheeling, IL). Bone micro-architecture was assessed in detail by micro-computed tomography using a fan-beam-type tomograph (μCT 40, Scanco Medical AG, Bassersdorf, Switzerland) and directed three-dimensional morphometry.

Statistical Analysis

Means were compared by using one-way analysis of Variance (ANOVA) or Students *t* test. $P < 0.05$ was considered as significant.

brn3a

S69350 Mus sp. class V POU transcription factor (Brn3a) gene, complete cds.

```
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 121 atgaacagca agcagcctca ctttgccatg catcccaccc tccctgagca caagtacccg
 181 tcgctgcact ccagctccga ggccatccgg cgggcctgcc tgcccacgcc gccggtaagc
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 361 ccgctgccag gcacgcgtgg cccggggccg ctggaggccc gggtcccatc cgcctgtgcc
 421 tctgtccagc gcctgccatc cgcggggagc tctcgggccg cggctgtcga cttggctcca
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Human POU domain factor (Brn-3a) gene, exon 1.

ACCESSION U10062 U09783

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 121 ccg  (SEQ ID NO:32)
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Human POU daomain factor (Brn-3a) gene, exon 2, complete cds.

ACCESSION U10063 U09783

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1141 acttactga  (SEQ ID NO:33)
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The ORF is nucleotides 1-123 of exon 1 and nucleotides 1-1149 of exon 2.

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brn3b

S69351 Mus musculus BRN-3b (Brn-3b) gene, complete cds.

```
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1021 atgtggtgcg cgtctggttc tgcaaccaga ggcagaaaca gaagaaggtg aaatactctg
1081 ccggcattta g  (SEQ ID NO:35)
```

protein_id=AAB30578.1; db_xref=GI:546434

MCAFYLQLQSNIFGGLDESLLARAEALAAVDIVSQSKSHHHHPPHHSPFKPDATYHTMNTIPCTSAASSSSVPISHP
SALAGTHHHHHHHHHHHHQPHQALEGELLEHLSPGLALGAMAGPDGTVVSTPAHAPHMATMNPMHQAALSMAHAHGL
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LQAWLEEAEKSHREKLTKPELFNGAEKKRKRTSIAAPEKRSLEAYFAIQPRPSSEKIAAIAEKLDLKKNVVRVWFCN
QRQKQKKVKYSAGI (SEQ ID NO:36)

Human POU domain protein (Brn-3b) mRNA, complete cds.

ACCESSION U06233

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   1 agacctcggc acccgttcag actgacagca gaggcggcga aggagcgcgt agccgagatc
  61 aggcgtacag agtccggagg cggcggcggg tgagctcaac ttcgcacagc ccttcccagc
 121 tccagccccg gctggcccgg cacttctcgg agggtcccgg cagccgggac cagtgagtgc
 181 ctctacggac cagcgccccg gcgggcggga agatgatgat gatgtccctg aacagcaagc
 241 aggcgtttag catgccgcac ggcggcagcc tgcacgtgga gcccaagtac tcggcactgc
 301 acagcacctc gccgggctcc tcggctccca tcgcgccctc ggccagctcc cccagcagct
 361 cgagcaacgc tggtggtggc ggcggcggcg gcggcggcgg cggcggcggc ggcggaggcc
 421 gaagcagcag ctccagcagc agtggcagca gcggcggcgg gggctcggag gctatgcgga
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2761 gcttgtaaat tcacattgtt tggaaaaatt cttttggaac aaaaaattag gtacatgata
2821 actggtacct tatctactgt aaatatttca ttaaaaatga tgcacacata gatatattct
2881 tacaaatttt gctgtattgc tgttctcttt gaggctctcc aaagtcttga gttctgtata
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3001 atttttggtg ttgttcgatt gtctttcatt gaagagataa ttttaatgtt ttattggcaa
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NO:37)
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The ORF is nucleotides 213-1445.
protein_id=AAA16509.1; db_xref="GI:458391"

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SHNNMIALKPILQAWLEEAEKSHREKLTKPELFNGAEKKRKRTSIAAPEKRSLEAYFAIQPRPSSEKIAAIAEKLDL
KKNVVRVWFCNQRQKQKRMKYSAGI  (SEQ ID NO:38)
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brn3c

S69352 Mus musculus BRN-3c (Brn-3c) gene, complete cds.

```
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  61 tagcaagacg agcttgattc catgtccccc gctgcctccc tgccagactc ccgaagatga
```

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 121 tggccatgaa cgccaagcac cgtttcggca tgcaccccgt actgcaagaa cccaaattct
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 661 cccacggcaa gaaccatccg ttcaagcccg acgccaccta ccataccatg agcagcgtgc
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1501 aggagcctgg agagcctaat gcatcgcccc cttccgatgg gaggggagct tacgggacac
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protein_id=AAB30579.1; db_xref=GI:546436

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ESLTLSHNNMIALKPVLQAWLEEAEAAYREKNSKPELFNGSERKRKRTSIAAPEKRSLEAYFAIQPRPSSEKIAAIA
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Human POU domain factor (Brn-3c) gene, exon 1.

ACCESSION U10060 U09718

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   1 atgatggcca tgaactccaa gcagcctttc ggcatgcacc cggtgctgca agaacccaaa
  61 ttctccagtc tgcactctgg ctccgaggct atgcgccgag tctgtctccc agccccgcag
(SEQ ID NO:41)
```

Human POU domain factor (Brn-3c) gene, exon 2, complete cds.

ACCESSION U10061 U09718

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 601 atgatcgctc tcaagccggt gctccaggcc tggttggagg aggccgaggc cgcctaccga
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```
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      781 tctgagaaga tcgcggccat cgctgagaaa ctggacctta aaaagaacgt ggtgagagtc
      841 tggttctgca accagagaca gaaacagaaa cgaatgaagt attcggctgt ccactga
(SEQ ID NO:42)
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The ORF is nucleotides 1-120 of exon 1 and nucleotides 1-897 of exon 2.

protein_id=AAA57160.1; db_xref=GI:602102

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.....

The present invention features methods of identifying a bone resorption disease or a bone generating disease, methods for prognosing and/or diagnosing a bone resorption disease or a bone generating disease, methods for identifying a compound that modulates bone resorption disease development or bone generating disease development, methods for determining the efficacy of a bone resorption disease therapy or a bone generating disease therapy, and oligonucleotide microarrays containing probes for genes involved in osteoclast development.

Accordingly, the invention features a method of identifying a bone resorption disease comprising determining a gene expression profile from a gene expression product of at least one bone resorption disease informative gene having increased expression in osteoclasts in a sample derived from bone tissue relative to a control. Increased expression of the gene expression product in the sample is indicative of a bone resorption disease.

The invention features a method of identifying a bone resorption disease comprising determining a gene expression profile from a gene expression product of at least one bone resorption disease informative gene having decreased expression in osteoclasts in a sample derived from bone tissue relative to a control. Decreased expression of the gene expression product in the sample is indicative of a bone resorption disease.

The invention features a method of identifying a bone generating disease comprising determining a gene expression profile from a gene expression product of at least one bone resorption disease informative gene having increased expression in osteoclasts in a sample

derived from bone tissue relative to a control. Decreased expression of the gene expression product in the sample is indicative of a bone generating disease.

The invention features a method of identifying a bone generating disease comprising determining a gene expression profile from a gene expression product of at least one bone resorption disease informative gene having decreased expression in osteoclasts in a sample derived from bone tissue relative to a control. Increased expression of the gene expression product in the sample is indicative of a bone generating disease.

The invention features a method of identifying a Compound for use in decreasing bone resorption disease development, comprising the steps of contacting a cell or cell lysate sample with a candidate compound; and detecting an increase in expression of at least one informative gene having decreased expression in osteoclasts. A candidate compound that increases the expression of the informative gene is a compound for use in decreasing bone resorption disease development.

The invention features a method of identifying a compound for use in decreasing bone resorption disease development, comprising the steps of contacting a cell or cell lysate sample with a candidate compound; and detecting a decrease in expression of at least one informative gene having increased expression in osteoclasts. A candidate compound that decreases the expression of the informative gene is a compound for use in decreasing bone resorption disease development.

The invention features a method of identifying a compound for use in decreasing bone generating disease development, comprising the steps of contacting a cell or cell lysate sample with a candidate compound; and detecting a decrease in expression of at least one informative gene having decreased expression in osteoclasts. A candidate compound that decreases the expression of the informative gene is a compound for use in decreasing bone generating disease development.

The invention features a method of identifying a compound for use in decreasing bone generating disease development, comprising the steps of contacting a cell or cell lysate sample with a candidate compound; and detecting an increase in expression of at least one informative gene having increased expression in osteoclasts. A candidate compound that increases the expression of the informative gene is a compound for use in decreasing bone generating disease development.

The above-described compound screening methods can be carried out by detecting a change in the biological activity of an informative gene expression product as an indication that the candidate compound is useful for decreasing development of a bone resorption disease or a bone generating disease.

Screens can be carried out for compounds that further increase the expression of a gene or the biological activity of a gene expression product already overexpressed in a bone resorption disease or a bone generating disease, or that further decrease the expression of a gene or the biological activity of a gene expression product already underexpressed in a bone resorption disease or a bone generating disease. These compounds can be identified according the screening methods described herein. These compounds should be avoided during treatment regimens for a bone resorption disease or a bone generating disease. For example, a compound that further increases the expression of a gene or the biological activity of a gene expression product already overexpressed in a bone resorption disease, or that further decreases the expression of a gene or the biological activity of a gene expression product already underexpressed in a bone resorption disease can be identified as a compound that should not be administered to a patient with a bone resorption disease. In addition, a compound that further increases the expression of a gene or the biological activity of a gene expression product already overexpressed in a bone generating disease, or that further increases the expression of a gene or the biological activity of a gene expression product already underexpressed in a bone generating disease can be identified as a compound that should not be administered to a patient with a bone generating disease.

The invention features a method of identifying a polypeptide that interacts with an informative gene expression product having increased or decreased expression in osteoclasts, a bone resorption disease, or a bone generating disease in a yeast two-hybrid system. The method comprises providing a first nucleic acid vector comprising a nucleic acid molecule encoding a DNA binding domain and a polypeptide encoded by the informative gene that is increased or decreased in osteoclasts, a bone resorption disease, or a bone generating disease; providing a second nucleic acid vector comprising a nucleic acid encoding a transcription activation domain and a nucleic acid encoding a test polypeptide; contacting the first nucleic acid vector with the second nucleic acid vector in a yeast two-hybrid system; and assessing transcriptional activation in the yeast two-hybrid system. An increase in transcriptional activation relative to a control indicates that the test polypeptide is a polypeptide that interacts with the informative gene

expression product having increased or decreased expression in osteoclasts, a bone resorption disease, or a bone generating disease.

The invention also relates to compounds identified according to the above-described screening methods. Such compounds can be used to treat a bone resorption disease or a bone generating disease, as appropriate.

The invention features a method of predicting the likelihood of bone resorption disease development in a subject, comprising determining a gene expression profile from a gene expression product of at least one informative gene having increased expression in osteoclasts in a sample derived from bone tissue of a subject relative to a control. Increased expression of the gene in the sample indicates an increased likelihood of bone resorption disease development in the subject.

The invention features a method of predicting the likelihood of bone resorption disease development in a subject, comprising determining a gene expression profile from a gene expression product of at least one informative gene having decreased expression in osteoclasts in a sample derived from bone tissue of a subject relative to a control. Decreased expression of the gene in the sample indicates an increased likelihood of bone resorption disease development in the subject.

The invention features a method of predicting the likelihood of bone generating disease development in a subject, comprising determining a gene expression profile from a gene expression product of at least one informative gene having increased expression in osteoclasts in a sample derived from bone tissue of a subject relative to a control. Decreased expression of the gene in the sample indicates an increased likelihood of bone generating disease development in the subject.

The invention features a method of predicting the likelihood of bone generating disease development in a subject, comprising determining a gene expression profile from a gene expression product of at least one informative gene having decreased expression in osteoclasts in a sample derived from bone tissue of a subject relative to a control. Increased expression of the gene in the sample indicates an increased likelihood of bone generating disease development in the subject.

The invention features a method of diagnosing a bone resorption disease in a subject, comprising determining a gene expression profile from a gene expression product of at least one

informative gene having increased expression in osteoclasts in a sample derived from bone tissue of a subject relative to a control. Increased expression of the gene in the sample indicates the presence of a bone resorption disease in the subject.

The invention features a method of diagnosing a bone resorption disease in a subject, comprising determining a gene expression profile from a gene expression product of at least one informative gene having decreased expression in osteoclasts in a sample derived from bone tissue of a subject relative to a control. Decreased expression of the gene in the sample indicates the presence of a bone resorption disease in the subject.

The invention features a method of diagnosing a bone generating disease in a subject, comprising determining a gene expression profile from a gene expression product of at least one informative gene having increased expression in osteoclasts in a sample derived from bone tissue of a subject relative to a control. Decreased expression of the gene in the sample indicates the presence of a bone generating disease in the subject.

The invention features a method of diagnosing a bone generating disease in a subject, comprising determining a gene expression profile from a gene expression product of at least one informative gene having decreased expression in osteoclasts in a sample derived from bone tissue of a subject relative to a control. Increased expression of the gene in the sample indicates the presence of a bone generating disease in the subject.

The invention features a method of assessing efficacy of treatment of a bone resorption disease in a subject comprising the steps of determining a gene expression profile from a gene expression product of at least one informative gene having increased expression in osteoclasts in a sample derived from bone tissue of a subject relative to a control, and repeating the above step at one or more time points during treatment. Decreased expression of the gene in the sample over time indicates an effective treatment.

The invention features a method of assessing efficacy of treatment of a bone resorption disease in a subject comprising the steps of determining a gene expression profile from a gene expression product of at least one informative gene having increased expression in osteoclasts in a sample derived from bone tissue from a subject relative to a control, and repeating the above step at one or more time points during treatment. Increased expression or lack of decreased expression of the gene in the sample over time indicates a less effective treatment.

The invention features a method of assessing efficacy of treatment of a bone resorption disease in a subject comprising the steps of determining a gene expression profile from a gene expression product of at least one informative gene having decreased expression in osteoclasts in a sample derived from bone tissue of a subject relative to a control, and repeating the above step at one or more time points during treatment. Increased expression of the gene in the sample over time indicates an effective treatment.

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The invention features a method of assessing efficacy of treatment of a bone generating disease in a subject comprising the steps of determining a gene expression profile from a gene expression product of at least one informative gene having increased expression in osteoclasts in a sample derived from bone tissue of a subject relative to a control, and repeating the above step at one or more time points during treatment. Increased expression of the gene in the sample over time indicates an effective treatment.

The invention features a method of assessing efficacy of treatment of a bone generating disease in a subject comprising the steps of determining a gene expression profile from a gene expression product of at least one informative gene having increased expression in osteoclasts in a sample derived from bone tissue of a subject relative to a control, and repeating the above step at one or more time points during treatment. Decreased expression or lack of increased expression of the gene in the sample over time indicates a less effective treatment.

The invention features a method of assessing efficacy of treatment of a bone generating disease in a subject comprising the steps of determining a gene expression profile from a gene expression product of at least one informative gene having decreased expression in osteoclasts in a sample derived from bone tissue of a subject relative to a control, and repeating the above step at one or more time points during treatment. Decreased expression of the gene in the sample over time indicates an effective treatment.

The invention features a method of assessing efficacy of treatment of a bone resorption disease in a subject comprising the steps of determining a gene expression profile from a gene expression product of at least one informative gene having decreased expression in osteoclasts relative to a control, and repeating the above step at one or more time points during treatment. Increased expression or a lack of decreased expression of the gene in the sample over time indicates a less effective treatment.

The gene expression product is DNA or mRNA. In another embodiment, the gene expression profile is determined utilizing specific hybridization probes. The expression profile can be determined, for example, using oligonucleotide microarrays. In another embodiment, the gene expression product is a polypeptide. When the gene expression product is a polypeptide, the gene expression profile can be determined, for example, utilizing antibodies.

The invention features a solid substrate having immobilized thereon a plurality of detection agents specific for one or more informative genes selected from the group consisting of the genes in FIGs. 1A-1E and 2A-2B. In one embodiment, the solid substrate is a microarray. In another embodiment, the detection agents are a plurality of oligonucleotide probes specific for one or more informative genes selected from the group consisting of the genes in FIGs. 1A-1E and 2A-2B. In still another embodiment, the detection agents are a plurality of gene expression products encoded by one or more informative genes selected from the group consisting of the genes in FIGs. 1A-1E and 2A-2B.

The present invention relates to one or more osteoclast markers or sets of osteoclast markers or informative genes (also referred to as osteoclast associated genes) whose expression correlates with a distinction between samples. In a particular embodiment, the distinction is a distinction between the presence or absence of bone resorbing activity or bone generating activity, or the presence or absence of a bone resorption disease or a bone generating disease in a patient from which the sample was obtained. The distinction can also be efficacy of treatment.

Osteoclast markers are listed in Figures 1A-1E and in Figures 2A-2B. Osteoclast markers include MIP-1γ, CCR1, MIP-1α, RANTES, brn3a, brn3b, and brn3c.

An "informative gene" is a gene that can be used to classify a sample. An informative gene can be, for example, a gene that is differentially expressed in samples that are compared. Genes that are increased or decreased in expression in macrophages that have been stimulated to undergo differentiation into osteoclasts compared to unstimulated macrophages are examples of

informative genes, and are also referred to herein as "bone resorption disease informative genes." Informative genes that are osteoclast markers for use in the present invention are those shown in Figures 1A-1E (informative genes with increased expression in osteoclasts) and Figures 2A-2B (informative genes with decreased expression in osteoclasts). Particular informative genes are MIP-1γ, CCR1, MIP-1α, RANTES, brn3a, brn3b, and brn3c.

"Bone resorbing activity" means a body process through which bone is removed from the body, resulting in a decrease in bone mass and/or bone structure. Osteoclasts are the cells in the body responsible for bone resorption. In the body, bone resorbing activity is counteracted by "bone generating activity," a process by which new bone is formed by osteoblasts.

Too much bone resorbing activity, caused, for example, by the presence of too many osteoclasts, increased osteoclast activity, too few osteoblasts, and/or decreased osteoblast activity can lead to the development (initiation or progressions) of a "bone resorption disease," in which bone mass decreases below normal levels, resulting in structural deterioration of bone tissue. Bone mass can be measured using standard tests, for example, bone mineral density tests. Examples of bone resorption diseases include osteoporosis, hyperparathyroidism, Paget's disease, inflammatory conditions, rheumatoid arthritis, osteoarthritis, and periodontitis.

Insufficient levels of bone resorbing activity, caused, for example, by an insufficient number of osteoclasts, insufficient osteoclast activity, too many osteoblasts, and/or too much osteoblast activity, can result in development (initiation or progressions) of a "bone generating disease," in which bone mass increases above normal levels or in which bones are not remodeled by osteoclasts. Examples of bone generating diseases include osteopetrosis, axial osteosclerosis, and Osteopathia striata.

The level of expression of osteoclast markers is determined to classify a sample as to the presence or absence of bone resorbing activity or bone generating activity or the presence or absence of a bone resorption disease or a bone generating disease in the patient from which the sample was obtained. Genes having increased expression in differentiated osteoclasts or in cells undergoing differentiation into osteoclasts are compared to those in cells with the potential to differentiate into osteoclasts, but which have not undergone differentiation, or cells having decreased expression in differentiated osteoclasts or in cells differentiating into osteoclasts are compared to those in cells with the potential to differentiate into osteoclasts, but which have not undergone differentiation. Such osteoclast markers or bone disease informative genes can be, for

example, all or a subset of the genes shown in Figures 1A-1E and Figures 2A-2B. Figures 1A-1E show informative genes (*i.e.*, osteoclast markers) whose expression is increased in RAW 264.7 macrophage-like cells stimulated with Receptor Activator of NFκB Ligand (RANKL) and normal mouse bone marrow macrophages stimulated with Macrophage-Colony Stimulating Factor (M-CSF) and RANKL, compared to unstimulated cells (control). Figures 2A-2B show informative genes whose expression is decreased in RAW 264.7 macrophage-like cells stimulated with Receptor Activator of NFκB Ligand (RANKL) and normal mouse bone marrow macrophages stimulated with Macrophage-Colony Stimulating Factor (M-CSF) and RANKL, compared to unstimulated cells (control).

The invention also relates to methods of diagnosing or predicting the likelihood of development of a bone resorption disease or a bone generating disease in a patient comprising the steps of providing a sample, for example, from one or more cells (*e.g.*, bone cells and/or bone marrow); and determining a gene expression profile of at least one informative gene or determining the level of expression of an osteoclast marker in the sample, wherein the gene expression profile or the level of expression is correlated with the presence or absence of a bone resorption disease or a bone generating disease or an increased or decreased likelihood of developing a bone resorption disease or a bone generating disease.

"Gene expression products" are proteins, polypeptides, or nucleic acid molecules (e.g., mRNA, tRNA, rRNA, or cRNA) that result from transcription or translation of genes. The present invention can be effectively used to analyze proteins, peptides or nucleic acid molecules that are the result of transcription or translation. The nucleic acid molecule levels measured can be derived directly from the gene or, alternatively, from a corresponding regulatory gene or regulatory sequence element. All forms of gene expression products can be measured. Additionally, variants of genes and gene expression products including, for example, spliced variants and polymorphic alleles, can be measured. Similarly, gene expression can be measured by assessing the level of protein or derivative thereof translated from mRNA. The sample to be assessed can be any sample that contains a gene expression product. Suitable sources of gene expression products, *e.g.*, samples, can include intact cells, lysed cells, cellular material for determining gene expression, or material containing gene expression products.

Examples of such samples are brain, blood, bone marrow, plasma, lymph, urine, tissue, mucus, sputum, saliva or other cell samples. Methods of obtaining such samples are known in

the art. The sample can be bone marrow tissue or bone cells, such as osteogenic cells, osteoblasts, osteoclasts, osteocytes, and bone lining cells, as well as bone cell progenitors (*e.g.*, pre-osteoblasts and pre-osteoclasts *i.e.*, cells the are destined to become or can be stimulated to become osteoblasts or osteoclasts but do not yet have osteoblast or osteoclast biological activity) are used. In another preferred embodiment, bone marrow tissue is used.

In one embodiment, the gene expression product is a protein or polypeptide. The determination of the gene expression profile can be made using techniques for protein detection and quantitation known in the art. For example, antibodies specific for the protein or polypeptide can be obtained using methods, which are routine in the art, and the specific binding of such antibodies to protein or polypeptide gene expression products can be detected and measured. Alternatively, the biological activity of the gene expression product can be measured as an indicator of the gene expression profile.

The gene expression product can be mRNA and the gene expression levels are obtained, *e.g.*, by contacting the sample with a suitable microarray, and determining the extent of hybridization of the nucleic acid in the sample to the probes on the microarray.

Using the methods described herein, expression of numerous osteoclast markers can be measured simultaneously to determine an expression profile. The assessment of numerous genes provides for a more accurate evaluation of the sample because there are more osteoclast markers or informative genes that can assist in classifying the sample.

"Expression profile" or "gene expression profile" is the level or amount of expression of osteoclast markers or informative genes as assessed by methods described herein. The expression profile can comprise data for one or more osteoclast markers or informative genes and can be measured at a single time point or over a period of time.

Gene expression profiles can be determined using various methods known in the art. For example, gene expression levels can be measured or assessed and assigned a value that is obtained from an apparatus that can measure gene expression levels. Gene expression levels refer to the amount of expression of the gene expression product, as described herein. The values are raw values from the apparatus, or values that are optionally rescaled, filtered and/or normalized. Such data is obtained, for example, from a GENECHIP® probe array or Microarray (Affymetrix, Inc.) (U.S. Patent Nos. 5,631,734, 5,874,219, 5,861,242, 5,858,659, 5,856,174, 5,843,655, 5,837,832, 5,834,758, 5,770,722, 5,770,456, 5,733,729, and 5,556,752, all of which

are incorporated herein by reference in their entirety), and the expression levels are calculated with software (e.g., Affymetrix GENECHIP® software). Nucleic acids (e.g., mRNA) from a sample which has been subjected to particular stringency conditions hybridize to the probes on the chip. The nucleic acid to be analyzed (e.g., the target) is isolated, amplified and labeled with a detectable label, (e.g., ^{32}P or fluorescent label) prior to hybridization to the arrays. Once hybridization occurs, the arrays are inserted into a scanner which can detect patterns of hybridization. The hybridization data are collected as light emitted from the labeled groups which is now bound to the probe array. The probes that perfectly match the target produce a stronger signal than those that have mismatches. Since the sequence and position of each probe on the array are known, by complementarity, the identity of the target nucleic acid applied to the probe is determined. Quantitation of gene profiles from the hybridization of labeled mRNA/DNA microarray can be performed by scanning the microarrays to measure the amount of hybridization at each position on the microarray with an Affymetrix scanner (Affymetrix, Santa Clara, CA).

For each stimulus, a time series of cDNA levels (C={C1,C2,C3,...Cn}) and a corresponding time series of mRNA levels (M={M1,M2,M3,...Mn}) in control medium in the same experiment as the stimulus is obtained. Quantitative data is then analyzed using methods known in the art. Microarrays are only one method of obtaining gene expression values. Other methods for obtaining gene expression values known in the art or developed in the future can be used with the present invention.

Once the gene expression values are prepared, the sample can be classified. Genes that can be used for classification (informative genes) have been identified as a result of work described herein and are shown in Figures 1A-1E and 2A-2B. Not all informative genes for a particular class distinction (e.g., bone resorbing activity, bone generating activity, a bone resorption disease, or a bone generating disease) must be assessed in order to classify a sample. For example, a subset of the informative genes which demonstrate a high correlation with a class distinction can be used. This subset can be, for example, one or more genes, for example, 2, 3, or 4 genes, 5 or more genes, for example, 6, 7, 8, or 9 genes, 10 or more genes, 25 or more genes, or more genes, or 50 or more genes. One subset is one or more of OC 1-285, brn3a, brn3b, and brn3c. Another subset is one or more of OC 286-364. Typically the accuracy of the classification will increase with the number of informative genes assessed.

The invention also provides methods (also referred to herein as "screening assays") for identifying agents or compounds (e.g., fusion proteins, polypeptides, peptidomimetics, prodrugs, receptors, binding agents, antibodies, small molecules or other drugs, or ribozymes) that alter or modulate (e.g., increase or decrease) the activity or expression of the osteoclast markers (*e.g.*, polypeptides encoded by the informative genes) as described herein, or that otherwise interact with the informative genes and/or polypeptides described herein. Such compounds can be compounds or agents that bind to informative gene expression products described herein (*e.g.*, the polypeptides encoded by the informative genes in Figures 1A-1E and 2A-2B), and that have a stimulatory or inhibitory effect on, for example, activity of the polypeptide encoded by an informative gene described herein; or that change (e.g., enhance or inhibit) the ability of a polypeptide encoded by an informative gene to interact with compounds or agents that bind such an informative gene polypeptide; or that alter post-translational processing of such a polypeptide (e.g., agents that alter proteolytic processing to direct the polypeptide from where it is normally synthesized to another location in the cell, such as the cell surface or the nucleus; or agents that alter proteolytic processing such that more polypeptide is released from the cell, etc.). The modulation can result in an increase or a decrease in the occurrence, severity, or progression of a bone resorption disease or a bone generating disease.

The candidate compound can cause an alteration in the activity of a polypeptide encoded by an informative gene of the present invention. For example, the activity of the polypeptide can be altered (increased or decreased) by at least 1.5-fold to 2-fold, at least 3-fold, or, at least 5-fold, relative to the control. Alternatively, the polypeptide activity can be altered, for example, by at least 10%, at least 20%, 40%, 50%, or 75%, or by at least 90%, relative to the control.

The invention provides assays for screening candidate compounds or test agents to identify compounds that bind to or modulate the activity of a polypeptide encoded by an informative gene described herein (or biologically active portion(s) thereof), as well as agents identifiable by the assays. A "candidate compound", "candidate substance" or "test agent" is a chemical molecule, be it naturally-occurring or artificially-derived, and includes, for example, peptides, proteins, synthetic molecules, for example, synthetic organic molecules, naturally-occurring molecules, nucleic acid molecules, and components thereof.

In general, candidate compounds for use in the present invention may be identified from large libraries of natural products or synthetic (or semi-synthetic) extracts or chemical libraries

according to methods known in the art. The precise source of test extracts or compounds is not critical to the screening procedure(s) of the invention. Accordingly, virtually any number of chemical extracts or compounds can be screened using the exemplary methods described herein. Examples of such extracts or compounds include, but are not limited to, plant-, fungal-, prokaryotic- or animal-based extracts, fermentation broths, and synthetic compounds, as well as modifications of existing compounds. Numerous methods are also available for generating random or directed synthesis (e.g., semi-synthesis or total synthesis) of any number of chemical compounds, including, but not limited to, saccharide-, lipid-, peptide-, and nucleic acid-based compounds. Synthetic compound libraries are commercially available, *e.g.*, from Brandon Associates (Merrimack, NH) and Aldrich Chemical (Milwaukee, WI). Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant, and animal extracts are commercially available from a number of sources, including Biotics (Sussex, UK), Xenova (Slough, UK), Harbor Branch Oceangraphics Institute (Ft. Pierce, FL), and PharmaMar, U.S.A. (Cambridge, MA). In addition, natural and synthetically produced libraries are generated, if desired, according to methods known in the art, e.g., by standard extraction and fractionation methods. For example, candidate compounds can be obtained using any of the numerous approaches in combinatorial library methods known in the art, including: biological libraries; spatially addressable parallel solid phase or solution phase libraries; synthetic library methods requiring deconvolution; the "one-bead one-compound" library method; and synthetic library methods using affinity chromatography selection. The biological library approach is limited to polypeptide libraries, while the other four approaches are applicable to polypeptide, non-peptide oligomer or small molecule libraries of compounds (Lam, Anticancer Drug Des., 12: 145 (1997)). Furthermore, if desired, any library or compound is readily modified using standard chemical, physical, or biochemical methods.

When a crude extract is found to modulate (i.e., increase or decrease) the expression and/or activity of the informative genes and/or their encoded polypeptides, further fractionation of the positive lead extract is necessary to isolate chemical constituents responsible for the observed effect. Thus, the goal of the extraction, fractionation, and purification process is the careful characterization and identification of a chemical entity within the crude extract having an activity that stimulates or inhibits nucleic acid expression, polypeptide expression, or polypeptide biological activity. The same assays described herein for the detection of activities in

mixtures of compounds can be used to purify the active component and to test derivatives thereof. Methods of fractionation and purification of such heterogeneous extracts are known in the art. If desired, compounds shown to be useful agents for treatment are chemically modified according to methods known in the art. Compounds identified as being of therapeutic value may be subsequently analyzed using animal models for diseases in which it is desirable to alter the activity or expression of the nucleic acids or polypeptides of the present invention.

To identify candidate compounds that alter the biological activity of a polypeptide encoded by an informative gene as described herein, a cell, tissue, cell lysate, tissue lysate, or solution containing or expressing a polypeptide encoded by the informative gene (e.g., a polypeptide encoded by a gene in any of Figures 1A-1E or 2A-2B), or a fragment or derivative thereof, can be contacted with a candidate compound to be tested under conditions suitable for biological activity of the polypeptide. Alternatively, the polypeptide can be contacted directly with the candidate compound to be tested. The level (amount) of polypeptide biological activity is assessed/measured, either directly or indirectly, and is compared with the level of biological activity in a control (i.e., the level of activity of the polypeptide or active fragment or derivative thereof in the absence of the candidate compound to be tested, or in the presence of the candidate compound vehicle only). If the level of the biological activity in the presence of the candidate compound differs, by an amount that is statistically significant, from the level of the biological activity in the absence of the candidate compound, or in the presence of the candidate compound vehicle only, then the candidate compound is a compound that alters the biological activity of the polypeptide encoded by an informative gene of the invention. For example, an increase in the level of polypeptide biological activity relative to a control, indicates that the candidate compound is a compound that enhances (is an agonist of) the polypeptide biological activity. Similarly, a decrease in the polypeptide biological activity relative to a control, indicates that the candidate compound is a compound that inhibits (is an antagonist of) the polypeptide biological activity.

The level of biological activity of an osteoclast marker that is a polypeptide encoded by an informative gene in the presence of the candidate compound to be tested, is compared with a control level that has previously been established. A level of polypeptide biological activity in the presence of the candidate compound that differs from (i.e., increases or decreases) the control

level by an amount that is statistically significant indicates that the compound alters the biological activity of the polypeptide.

The level and/or pattern of expression of an osteoclast marker that is an informative gene in the presence of the candidate compound to be tested, is compared with a control level and/or pattern of expression that has previously been established. A level and/or pattern of expression of an osteoclast marker that is an informative gene expression in the presence of the candidate compound that differs from the control level and/or pattern of expression by an amount or in a manner that is statistically significant indicates that the candidate compound alters osteoclast marker expression.

Compounds that alter the expression of an osteoclast marker, or that otherwise interact with an osteoclast marker described herein, can be identified using a cell, cell lysate, or solution containing a nucleic acid encoding the promoter region of an osteoclast marker that is an informative gene operably linked to a reporter gene. A "promoter" is a minimal nucleotide sequence sufficient to direct transcription, and "operably linked" means that a gene and one or more regulatory sequences are connected in such a way as to permit gene expression when the appropriate molecules (e.g., transcriptional activator proteins) are bound to the regulatory sequences. Examples of reporter genes and methods for operably linking a reporter gene to a promoter are known in the art. After contact with a candidate compound to be tested, the level of expression of the reporter gene (e.g., the level of mRNA or of protein expressed) is assessed, and is compared with the level of expression in a control (i.e., the level of expression of the reporter gene in the absence of the candidate compound, or in the presence of the candidate compound vehicle only). If the level of expression in the presence of the candidate compound differs by an amount or in a manner that is statistically significant from the level in the absence of the candidate compound, or in the presence of the candidate compound vehicle only, then the candidate compound is a compound that alters the expression of the informative gene, as indicated by its ability to alter expression of the reporter gene that is operably linked to the informative gene promoter. Enhancement of the expression of the reporter gene indicates that the compound is an agonist of the informative gene polypeptide activity. Similarly, inhibition of the expression of the reporter gene indicates that the compound is an antagonist of the informative gene polypeptide activity.

In another embodiment, the level of expression of the reporter in the presence of the candidate compound to be tested, is compared with a control level that has been established previously. A level in the presence of the candidate compound that differs from the control level by an amount or in a manner that is statistically significant indicates that the candidate compound alters informative gene expression.

The present invention also features methods of detecting and/or identifying a compound that alters the interaction between a polypeptide encoded by an informative gene and a polypeptide (or other molecule) with which the polypeptide normally interacts (*e.g.*, in a cell or under physiological conditions). In one example, a cell or tissue that expresses or contains a compound (e.g., a polypeptide or other molecule) that interacts with a polypeptide encoded by an informative gene (such a molecule is referred to herein as a "polypeptide substrate") is contacted with the informative gene polypeptide in the presence of a candidate compound, and the ability of the candidate compound to alter the interaction between the polypeptide encoded by the informative gene and the polypeptide substrate is determined, for example, by assaying activity of the polypeptide. Alternatively, a cell lysate or a solution containing the informative gene polypeptide, the polypeptide substrate, and the candidate compound can be used. A compound that binds to the informative gene polypeptide or to the polypeptide substrate can alter the interaction between the informative gene polypeptide and the polypeptide substrate by interfering with (inhibiting), or enhancing the ability of the informative gene polypeptide to bind to, associate with, or otherwise interact with the polypeptide substrate.

Determining the ability of the candidate compound to bind to the informative gene polypeptide or a polypeptide substrate can be accomplished, for example, by coupling the candidate compound with a radioisotope or enzymatic label such that binding of the candidate compound to the informative gene polypeptide or polypeptide substrate can be determined by directly or indirectly detecting the candidate compound labeled with ^{125}I, ^{35}S, ^{14}C, or ^{3}H, and the detecting the radioisotope (*e.g.*, by direct counting of radioemmission or by scintillation counting). Alternatively, the candidate compound can be enzymatically labeled with, for example, horseradish peroxidase, alkaline phosphatase, or luciferase, and the enzymatic label is then detected by determination of conversion of an appropriate substrate to product. In another alternative, one of the other components of the screening assay (*e.g.*, the polypeptide substrate or the informative gene polypeptide) can be labeled, and alterations in the interaction between the

informative gene polypeptide and the polypeptide substrate can be detected. In these methods, labeled unbound components can be removed (e.g., by washing) after the interaction step in order to accurately detect the effect of the candidate compound on the interaction between the informative gene polypeptide and the polypeptide substrate.

It is also within the scope of this invention to determine the ability of a candidate compound to interact with the informative gene polypeptide or polypeptide substrate without labeling of any of the interactants. For example, a microphysiometer can be used to detect the interaction of a candidate compound with a polypeptide encoded by an informative gene or a polypeptide substrate without labeling either the candidate compound, the polypeptide encoded by the informative gene, or the polypeptide substrate (McConnell et al., Science 257: 1906-1912 (1992)). A "microphysiometer" (e.g., CYTOSENSOR™) is an analytical instrument that measures the rate at which a cell acidifies its environment using a light-addressable potentiometric sensor (LAPS). Changes in this acidification rate can be used as an indicator of the interaction between ligand and polypeptide.

Assays can be used to identify polypeptides that interact with one or more polypeptides encoded by an informative gene. For example, a yeast two-hybrid system such as that described by Fields and Song (Fields and Song, Nature 340: 245-246 (1989)) can be used to identify polypeptides that interact with one or more polypeptides encoded by an informative gene.

In the above assay methods of the present invention, it may be desirable to immobilize a polypeptide encoded by an informative gene, or a polypeptide substrate, or other components of the assay on a solid support, in order to facilitate separation of complexed from uncomplexed forms of one or both of the polypeptides, as well as to accommodate automation of the assay. Binding of a candidate compound to the polypeptide, or interaction of the polypeptide with a polypeptide substrate in the presence and absence of a candidate compound, can be accomplished in any vessel suitable for containing the reactants. Examples of such vessels include microtitre plates, test tubes, and micro-centrifuge tubes. In one embodiment, a fusion protein (e.g., a glutathione-S-transferase fusion protein) can be provided that adds a domain that allows the informative gene polypeptide, or the polypeptide substrate to be bound to a matrix or other solid support.

This invention further pertains to novel compounds identified by the above-described screening assays. Accordingly, it is within the scope of this invention to further use a compound

identified as described herein in an appropriate animal model. For example, a compound identified as described herein can be used in an animal model to determine the efficacy, toxicity, or side effects of treatment with such a compound. Furthermore, this invention pertains to uses of novel compounds identified by the above-described screening assays for treatments as described herein. In addition, a compound identified as described herein can be used to alter activity of a polypeptide encoded by an informative gene, or to alter expression of the informative gene, by contacting the polypeptide or the nucleic acid molecule (or contacting a cell comprising the polypeptide or the nucleic acid molecule) with the compound identified as described herein.

Compounds identified according to the methods of the above-described screens can be used to modulate (increase or decrease (inhibit)) bone resorbing activity or bone generating activity. A candidate compound that decreases the expression of an informative gene with increased expression in osteoclasts or increases the expression of an informative gene with decreased expression in osteoclasts is a compound for use in decreasing or inhibiting bone resorbing activity in a subject or for treating a bone resorption disease. Such a compound is also useful for increasing bone generating activity in a subject. In addition, a candidate compound that increases the expression of an informative gene with increased expression in osteoclasts or that decreases the expression of an informative gene with decreased expression in osteoclasts is a compound for use in decreasing or inhibiting bone generating activity in a subject or for treating a bone generating disease. Such a compound is also useful for increasing bone resorbing activity in a subject.

The cell or cell lysate sample is derived from bone tissue. In another example, the sample comprises or consists of osteoclasts and/or osteoblasts. Such cells can be primary cells or cultured cells. In another embodiment, the informative genes having increased expression in osteoclasts are selected from the group consisting of the genes in Figures 1A-1E and the informative genes having decreased expression in osteoclasts are selected from the group consisting of the genes in Figures 2A-2B.

The present invention further relates to antibodies that specifically bind a polypeptide, preferably an epitope, of an informative gene product of the present invention (as determined, for example, by immunoassays, a technique well known in the art for assaying specific antibody-antigen binding). Antibodies of the invention include, but are not limited to, polyclonal,

monoclonal, multispecific, human, humanized or chimeric antibodies, single chain antibodies, Fab fragments, F(ab') fragments, fragments produced by a Fab expression library, anti-idiotypic (anti-Id) antibodies (including, for example, anti-Id antibodies to antibodies of the invention), and epitope-binding fragments of any of the above.

An "antibody," refers to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, and more specifically, molecules that contain an antigen binding site that specifically binds an antigen. The immunoglobulin molecules of the invention can be of any type (for example, IgG, IgE, IgM, IgD, IgA and IgY), and of any class (for example, IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2) or subclass of an immunoglobulin molecule.

The antibodies can be antigen-binding antibody fragments and include, without limitation, Fab, Fab' and $F(ab')_2$, Fd, single-chain Fvs (scFv), single chain antibodies, disulfide-linked Fvs (sdFv) and fragments comprising either a V_L or V_H domain. Antigen-binding antibody fragments, including single-chain antibodies, can comprise the variable region(s) alone or in combination with the entirety or a portion of one or more of the following: hinge region, CH1, CH2, and CH3 domains. Also included in the invention are antigen-binding fragments also comprising any combination of variable region(s) with a hinge region, CH1, CH2, and/or CH3 domains.

The antibodies of the invention may be from any animal origin including birds and mammals. Preferably, the antibodies are human, murine, donkey, sheep, rabbit, goat, guinea pig, hamster, horse, or chicken.

"Human" antibodies include antibodies having the amino acid sequence of a human immunoglobulin and include antibodies produced by human B cells, or isolated from human sera, human immunoglobulin libraries or from animals transgenic for one or more human immunoglobulins and that do not express eridogenous immunoglobulins, as described in U.S. Pat. No. 5,939,598 by Kucherlapati et al., for example.

The antibodies of the present invention may be monospecific, bispecific, trispecific or of greater multispecificity. Multispecific antibodies may be specific for different epitopes of a polypeptide of the present invention or may be specific for both a polypeptide of the present invention as well as for a heterologous epitope, such as a heterologous polypeptide or solid support material.

Antibodies of the present invention may be described or specified in terms of the epitope(s) or portion(s) of a polypeptide of the present invention that they recognize or specifically bind. The epitope(s) or polypeptide portion(s) may be specified, for example, by N-terminal and/or C-terminal positions, or by size in contiguous amino acid residues. Antibodies that specifically bind any epitope or polypeptide encoded by an informative gene of the present invention may also be excluded. Therefore, the present invention includes antibodies that specifically bind a polypeptide encoded by an informative gene of the present invention, and allows for the exclusion of the same.

An "epitope," is a portion of a polypeptide which contacts an antigen-binding site(s) of an antibody or T cell receptor. Specific binding of an antibody to an antigen having one or more epitopes excludes non-specific binding to unrelated antigens, but does not necessarily exclude cross-reactivity with other antigens with similar epitopes.

Antibodies of the present invention may also be described or specified in terms of their cross-reactivity. Antibodies of the present invention may not display any cross-reactivity, such that they do not bind any other analog, ortholog, or homolog of a polypeptide of the present invention. Alternatively, antibodies of the invention can bind polypeptides with at least about 95%, 90%, 85%, 80%, 75%, 70%, 65%, 60%, 55%, or 50% identity (as calculated using methods known in the art) to a polypeptide encoded by an informative gene of the present invention. Further included in the present invention are antibodies that bind polypeptides encoded by informative genes that hybridize to an informative gene of the present invention under stringent hybridization conditions, as will be appreciated by one of skill in the art.

Antibodies of the present invention can also be described or specified in terms of their binding affinity to a polypeptide of the invention. Preferred binding affinities include those with a dissociation constant or Kd less than $5x10^{-6}$ M, 10^{-6} M, $5x10^{-7}$ M, 10^{-7} M, $5x10^{-8}$ M, 10^{-8} M, $5x10^{-9}$ M, 10^{-9} M, $5x10^{-10}$ M, 10^{-10} M, $5x10^{-11}$ M, 10^{-11} M,$5x10^{-12}$ M, 10^{-11} M, $5x10^{-13}$ M, 10^{-13} M, $5x10^{-14}$ M, 10^{-14} M, $5x10^{-15}$ M, or 10^{-15} M.

The invention also provides antibodies that competitively inhibit binding of an antibody to an epitope of a polypeptide of the invention, as determined by any method known in the art for determining competitive binding, for example, using immunoassays. In particular embodiments, the antibody competitively inhibits binding to the epitope by at least about 90%, 80%, 70%, 60%, or 50%.

Antibodies of the present invention can act as agonists or antagonists of polypeptides encoded by the informative genes of the present invention. For example, the present invention includes antibodies which disrupt interactions with the polypeptides encoded by the informative genes of the invention either partially or fully. The invention also includes antibodies that do not prevent binding, but prevent activation or activity of the polypeptide. Activation or activity (for example, signaling) may be determined by techniques known in the art. Also included are antibodies that prevent both binding to and activity of a polypeptide encoded by an informative gene. Likewise included are neutralizing antibodies.

Antibodies of the present invention may be used, for example, and without limitation, to purify, detect, and target the polypeptides encoded by the informative genes described herein, including both in vitro and in vivo diagnostic and therapeutic methods. For example, the antibodies have use in immunoassays for qualitatively and quantitatively measuring levels of the polypeptides in biological samples. See, for example, Harlow et al., Antibodies: A Laboratory Manual (Cold Spring Harbor Laboratory Press, 2nd ed. 1988).

The antibodies of the present invention may be used either alone or in combination with other compositions. The antibodies may further be recombinantly fused to a heterologous polypeptide at the N— and/or C-terminus or chemically conjugated (including covalent and non-covalent conjugations) to polypeptides or other compositions. For example, antibodies of the present invention may be recombinantly fused or conjugated to molecules useful as labels in detection assays, or effector molecules such as heterologous polypeptides, drugs, or toxins.

The antibodies of the invention include derivatives that are modified, for example, by the covalent attachment of any type of molecule to the antibody such that covalent attachment does not prevent the antibody from recognizing its epitope. For example, but not by way of limitation, the antibody derivatives include antibodies that have been modified by glycosylation, acetylation, pegyllation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, or linkage to a cellular ligand or other protein. Any of numerous chemical modifications can be carried out by known techniques, including, but not limited to, specific chemical cleavage, acetylation, formylation, and metabolic synthesis of tunicamycin. Additionally, the derivative can contain one or more non-classical amino acids.

The antibodies of the present invention can be generated by any suitable method known in the art. For example, a polypeptide of the invention can be administered to various host

animals including, but not limited to, rabbits, mice, rats, or the like, to induce the production of sera containing polyclonal antibodies specific for the antigen. Various adjuvants can be used to increase the immunological response, depending on the host species, and include, but are not limited to, Freund's adjuvant (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (Bacille Calmette-Guerin) and corynebacterium parvum. Such adjuvants are well known in the art.

Monoclonal antibodies can be prepared using a wide variety of techniques also known in the art, including hybridoma cell culture, recombinant, and phage display technologies, or a combination thereof. "Monoclonal antibodies" are not necessarily limited to antibodies produced through hybridoma technology, but also refer to antibodies that are derived from a single clone, including any eukaryotic, prokaryotic, or phage clone.

Human antibodies are desirable for therapeutic treatment of human patients. These antibodies can be made by a variety of methods known in the art including phage display methods using antibody libraries derived from human immunoglobulin sequences. Human antibodies can also be produced using transgenic mice that are incapable of expressing functional endogenous immunoglobulins, but which can express human immunoglobulin genes. The transgenic mice are immunized with a selected antigen, for example, all or a portion of a polypeptide of the invention. Monoclonal antibodies directed against the antigen can be obtained from the immunized, transgenic mice using conventional hybridoma technology. The human immunoglobulin transgenes harbored by the transgenic mice rearrange during B cell differentiation, and subsequently undergo class switching and somatic mutation. Thus, using such a technique, it is possible to produce therapeutically useful IgG, IgA, IgM and IgE antibodies. For a detailed discussion of this technology for producing human antibodies and human monoclonal antibodies and protocols for producing such antibodies, see, for example, PCT publications WO 98/24893; WO 96/34096; WO 96/33735; and U.S. Patent Nos. 5,413,923; 5,625,126; 5,633,425; 5,569,825; 5,661,016; 5,545,806; 5,814,318; and 5,939,598.

Antibodies to the polypeptides encoded by the informative genes as described herein can, in turn, be utilized to generate anti-idiotype antibodies that "mimic" polypeptides of the invention using techniques well known to those skilled in the art. (*See, for example*, Greenspan

& Bona, FASEB J. 7(5):437-444 (1989) and Nissinoff, J. Immunol. 147(8):2429-2438 (1991)). For example, antibodies that bind to and competitively inhibit polypeptide multimerization and/or binding of a polypeptide to a ligand can be used to generate anti-idiotypes that "mimic" the polypeptide multimerization and/or binding domain and, as a consequence, bind to and neutralize polypeptide and/or its ligand. Such neutralizing anti-idiotypes or Fab fragments of such anti-idiotypes can be used in therapeutic regimens to neutralize polypeptide ligand. For example, such anti-idiotypic antibodies can be used to bind a polypeptide encoded by an informative gene and/or to bind its ligands, and thereby block its biological activity.

The antibodies or fragments thereof of the present invention can be fused to marker sequences, such as a peptide to facilitate their purification. For example, marker amino acid sequences include a hexa-histidine peptide, an HA tag, or a FLAG tag.

The present invention further encompasses antibodies or fragments thereof conjugated to a diagnostic or therapeutic agent. The antibodies can be used diagnostically, for example, to monitor the development or progression of a bone resorption or bone generation disease or disorder as part of a clinical testing procedure to determine the efficacy of a given treatment regimen. Detection can be facilitated by coupling the antibody to a detectable substance. Examples of detectable substances include enzymes (such as, horseradish peroxidase, alkaline phosphatase, beta-galactosidase, or acetyicholinesterase), prosthetic groups (such as streptavidinlbiotin and avidin/biotin), fluorescent materials (such as umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin), luminescent materials (such as luminol), bioluminescent materials (such as luciferase, luciferin, and aequorin), radioactive materials (such as, ^{125}I ^{131}I, ^{111}In or ^{99}Tc), and positron emitting metals using various positron emission tomographies, and nonradioactive paramagnetic metal ions.

An antibody or fragment thereof can be conjugated to a therapeutic moiety such as a bisphosphonate (e.g., alendronate, risedronate, pamidronate, etidronate, or tiludronate), calcitonin, estrogen, or an estrogen modulator.

Antibodies of the invention can also be attached to solid supports. These are particularly useful for immunoassays or purification of the target antigen. Such solid supports include, but are not limited to, glass, cellulose, silicon, polyacrylamide, nylon, polystyrene, polyvinyl chloride or polypropylene. Techniques for conjugating such therapeutic moiety to antibodies are

well known in the art, see, for example, Anion et al., "Monoclonal Antibodies For Immunotargeting Of Drugs In Cancer Therapy", in Monoclonal Antibodies And Cancer Therapy, Reisfeld et al. eds., pp. 243-56 (Alan R. Liss, Inc. 1985).

Alternatively, an antibody can be conjugated to a second antibody to form an antibody heteroconjugate as described by Segal in U.S. Patent No. 4,676,980.

An antibody of the invention, with or without conjugation to a therapeutic moiety, administered alone or in combination with an additional therapeutic agent, can be used as a therapeutic.

Antisense antagonists are also included in the present invention. Antisense technology can be used to control gene expression through antisense DNA or RNA, or through triple-helix formation. Antisense techniques are discussed for example, in Okano (J., Neurochem. 56:560 (1991)). The methods are based on binding of a polynucleotide to a complementary DNA or RNA. An antisense sequence can be generated internally by the organism, alternatively, the antisense sequence is separately administered.

The 5' coding portion of an informative gene can be used to design an antisense RNA oligonucleotide from about 10 to 40 base pairs in length. Generally, a DNA oligonucleotide is designed to be complementary to a region of the gene involved in transcription thereby preventing transcription and the production of the polypeptide. The antisense RNA oligonucleotide hybridizes to the mRNA in vivo and blocks translation of the mRNA molecule into polypeptide.

The antisense nucleic acid of the invention can be produced intracellularly by transcription from an exogenous sequence. For example, a vector or a portion thereof, is transcribed, producing an antisense nucleic acid of the invention. Such a vector contains the sequence encoding the antisense nucleic acid. The vector can remain episomal or become chromosomally integrated, as long as it can be transcribed to produce the desired antisense RNA. Vectors can be constructed by recombinant DNA technology and can be plasmid, viral, or otherwise, as is known in the art.

Expression can be controlled by any promoter known in the art to act in the target cells, such as vertebrate cells, and preferably human cells. Such promoters can be inducible or constitutive and include, without limitation, the SV40 early promoter region (Bernoist and Chambon, Nature 29:304-3 10 (1981), the promoter contained in the 3' long terminal repeat of

Rous sarcoma virus (Yamamoto et al., Cell 22:787-797 (1980)), the herpes thymidine promoter (Wagner et al., Proc. Nail Acad. Sci. U.S.A. 78:1441-1445 (1981)), and the regulatory sequences of the metallothionein gene (Brinster et al., Nature 296:39-42 (1982)).

The antisense nucleic acids of the invention comprise a sequence complementary to at least a portion of an RNA transcript of an informative gene. Absolute complementarity, although preferred, is not required. A sequence "complementary to at least a portion of an RNA," is a sequence having sufficient complementarity to be able to hybridize with the RNA, forming a stable duplex. The ability to hybridize will depend on both the degree of complementarity and the length of the antisense nucleic acid. Generally, the larger the hybridizing nucleic acid, the more base mismatches with the RNA it may contain and still form a stable duplex.

Oligonucleotides that are complementary to the 5' end of the RNA, for example, the 5' untranslated sequence up to and including the AUG initiation codon, are generally regarded to work most efficiently at inhibiting translation. However, sequences complementary to the 3' untranslated sequences of mRNAs have been shown to be effective at inhibiting translation of mRNAs as well. Thus, oligonucleotides complementary to either the 5'- or 3'-non-translated, non-coding regions of a nucleotide sequence can be used in an antisense approach to inhibit mRNA translation. Oligonucleotides complementary to the 5' untranslated region of the mRNA can include the complement of the AUG start codon. Antisense oligonucleotides complementary to mRNA coding regions can also be used in accordance with the invention. In one embodiment, the antisense nucleic acids are at least six nucleotides in length, and are preferably oligonucleotides ranging from about 6 to about 50 nucleotides in length. In other embodiments, the oligonucleotide is at least about 10, 17, 25 or 50 nucleotides in length.

The antisense oligonucleotides of the invention can be DNA or RNA, or chimeric mixtures, or derivatives or modified versions thereof, single-stranded or double-stranded. The oligonucleotide can be modified at the base moiety, sugar moiety, or phosphate backbone, for example, to improve stability of the molecule, hybridization, and the like. The oligonucleotide can include other appended groups such as peptides (for example, to target host cell receptors in vivo), or agents that facilitate transport across the cell membrane, or the blood-brain barrier, or intercalating agents.

The antisense oligonucleotide may comprise at least one modified base moiety which is selected from the group including, but not limited to, 5-fluorouracil, 5-bromouracil, 5-

chiorouracil, 5-iodouracil, hypoxanthine, xanthine, 4-acetylcytosine, 5-(carboxyhydroxylmethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-5 carboxymethylaminomethyluracil, dihydrouracil, a-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1 -methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, betaD-mannosylqueosine, 5' -methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosi ne, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine.

The antisense oligonucleotide may also comprise at least one modified sugar moiety selected from the group including, but not limited to, arabinose, 2-fluoroarabinose, xylulose, and hexose.

The antisense oligonucleotide can comprise at least one modified phosphate backbone selected from the group including, but not limited to, a phosphorothioate, a phosphorodithioate, a phosphoramidothioate, a phosphoramidate, a phosphordiamidate, a methyiphosphonate, an alkyl phosphotriester, and a formacetal or analog thereof.

Alternatively, the antisense oligonucleotide is an a-anomeric oligonucleotide. An cx-anomenc oligonucleotide forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual β-units, the strands run parallel to each other (Gautier et al., Nucl. Acids Res. 15:6625-664 1 (1987)). The oligonucleotide is a 2'-O-methylribonucleotide (Inoue et al., Nucl. Acids Res. 15:6131-6148 (1987)), or a chimeric RNA-DNA analog (Inoue et al., FEBS Lett. 2 15:327-330 (1987)).

Antisense oligonucleotides of the invention may be synthesized by standard methods known in the art, for example, by use of an automated DNA synthesizer.

Potential antagonists of the informative genes of the invention also include catalytic RNA, or a ribozyme. Hammerhead ribozymes cleave mRNAs at locations dictated by flanking regions that form complementary base pairs with the target mRNA. The target mRNA has the following sequence of two bases: 5'-UG-3'. The construction and production of hammerhead ribozymes is well known in the art and is described more fully in Haseloff and Gerlach (Nature 334:585-59 1 (1988)). Preferably, the ribozyme is engineered so that the cleavage recognition

site is located near the 5' end of the mRNA in order to increase efficiency and minimize the intracellular accumulation of non-functional mRNA transcripts.

Ribozymes of the invention can be composed of modified oligonucleotides (for example for improved stability or targeting). DNA constructs encoding the ribozyme can be under the control of a strong constitutive promoter, such as, for example, pol III or pol II promoter, so that a transfected cell will produce sufficient quantities of the ribozyme to destroy endogenous target mRNA and inhibit translation. Since ribozymes, unlike antisense molecules, are catalytic, a lower intracellular concentration is generally required for efficiency.

The present invention also provides pharmaceutical compositions, including both therapeutic and prophylactic compositions. Compositions within the scope of this invention include all compositions wherein the therapeutic agent, antibody, fragment or derivative, antisense oligonucleotide or ribozyme is contained in an amount effective to achieve its intended purpose. The effective dose is a function of a number of factors, including the specific antibody, the antisense oligonucleotide, antisense construct, ribozyme or polypeptide of the invention, the presence of a conjugated therapeutic agent, the patient and their clinical status.

The mode of administration may be by parenteral, subcutaneous, intravenous, intramuscular, intraperitoneal, transdermal, or buccal routes. Alternatively, or concurrently, administration may be orally. The dosage administered will be dependent upon the age, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired.

Such compositions generally comprise a therapeutically effective amount of a compound, and a pharmaceutically acceptable carrier. "Pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopoeia or other generally recognized pharmacopoeia for use in animals, and more particularly in humans. A "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the therapeutic is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water is a preferred carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour,

chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skimmed milk, glycerol, propylene, glycol, water, ethanol and the like. The composition can also contain minor amounts of wetting or emulsifying agents, and/or pH buffering agents.

These compositions can take the form of solutions, suspensions, emulsion, tablets, pills, capsules, powders, or sustained-release formulations. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides. Oral formulations can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, and the like. Such compositions will contain a therapeutically effective amount of the compound, preferably in purified form, together with a suitable amount of carrier so as to provide the form for proper administration to the patient. The formulation should suit the mode of administration.

The composition can be formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to a human. Typically, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the composition may also include a solubilizing agent and a local anesthetic such as lignocaine to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water free concentrate in a hermetically sealed container such as an ampoule or sachette indicating the quantity of active agent. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the composition is administered by injection, an ampoule of sterile water for injection or saline can be provided so that the ingredients may be mixed prior to administration.

The compounds of the invention can be formulated as neutral or salt forms. Pharmaceutically acceptable salts include those formed with anions such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, and the like, and those formed with cations such as those derived from sodium, potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

The compositions of the invention can be administered alone or in combination with other therapeutic agents. Therapeutic agents that can be administered in combination with the compositions of the invention, include but are not limited to chemotherapeutic agents, antibiotics, steroidal and non-steroidal anti-inflammatories, conventional immunotherapeutic

agents, cytokines and/or growth factors. Combinations may be administered either concomitantly, for example, as an admixture, separately but simultaneously or concurrently; or sequentially. This includes presentations in which the combined agents are administered together as a therapeutic mixture, and also procedures in which the combined agents are administered separately but simultaneously, for example, as through separate intravenous lines into the same individual. Administration "in combination" further includes the separate administration of one of the compounds or agents given first, followed by the second.

Conventional, nonspecific immunosuppressive agents, that may be administered in combination with the compositions of the invention include, but are not limited to, steroids, cyclosporine, cyclosporine analogs, cyclophosphamide methyiprednisone, prednisone, azathioprine, FK-506, 15-deoxyspergualin, and other immunosuppressive agents. Antibiotic agents that may be administered with the compositions of the invention include, but are not limited to, tetracycline, metronidazole, amoxicillin, betalactamases, aminoglycosides, macrolides, quinolones, fluoroquinolones, cephalosporins, erythromycin, ciprofloxacin, and streptomycin. Anti-inflammatory agents that can be administered with the compositions of the invention include, but are not limited to, glucocorticoids and the nonsteroidal anti-inflammatories, aminoarylcarboxylic acid derivatives, arylacetic acid derivatives, arylbutyric acid derivatives, arylcarboxylic acids, aryipropionic acid derivatives, pyrazoles, pyrazolones, salicylic acid derivatives, thiazinecarboxamides, e-acetamnidocaproic acid, S-adenosylmethionine, 3-amino-4-hydroxybutyric acid, amixetrine, bendazac, benzydamine, bucolome, difenpiramide, ditazol, emorfazone, guaiazulene, nabumetone, nimesulide, orgotein, oxaceprol, paranyline, perisoxal, pifoxime, proquazone, proxazole, and tenidap.

In another embodiment, compositions of the invention are administered in combination with another therapeutic agent for treating a bone resorption disease, or a bone generating disease. Therapeutic agents that may be administered with the compositions of the invention include, but are not limited to, a bisphosphonate (e.g., alendronate, risedronate, pamidronate, etidronate, or tiludronate), calcitonin, estrogen, and estrogen modulators.

The present invention is further directed to therapies which involve administering pharmaceutical compositions of the invention to an animal, preferably a mammal, and most preferably a human patient, for treating one or more of the described disorders. Therapeutic compositions of the invention include, for example, therapeutic agents identified in screening

assays, antibodies of the invention (including fragments, analogs and derivatives thereof as described herein), antisense oligonucleotides, ribozymes and nucleic acids encoding same. The compositions of the invention can be used to treat, inhibit, prognose, diagnose or prevent diseases, disorders or conditions associated with aberrant expression and/or activity of a polypeptide of the invention, including, but not limited to, any one or more of the diseases, disorders, or conditions such as, for example, a bone resorption disease or a bone generating disease.

The treatment and/or prevention of diseases and disorders associated with aberrant expression and/or activity of a polypeptide of the invention includes, but is not limited to, alleviating symptoms associated with those diseases and disorders. The amount of the compound of the invention which will be effective in the treatment, inhibition and prevention of a disease or disorder associated with aberrant expression and/or activity of a polypeptide of the invention can be determined by standard clinical techniques. In addition, *in vitro* assays may optionally be employed to help identify optimal dosage ranges. The precise dose to be employed in the formulation will also depend on the route of administration, and the seriousness of the disease or disorder. Effective doses may be extrapolated from dose-response curves derived from *in vitro* or animal model test systems.

For antibodies, the dosage administered to a patient is typically 0.1 mg/kg to 100 mg/kg of the patient's body weight. Preferably, the dosage administered to a patient is between 0.1 mg/kg and 20 mg/kg of the patient's body weight, more preferably 1 mg/kg to 10 mg/kg of the patient's body weight. Generally, human antibodies have a longer half-life within the human body than antibodies from other species due to the immune response to the foreign polypeptides. Thus, lower dosages of human antibodies and less frequent administration is often possible. Furthermore, the dosage and frequency of administration of antibodies of the invention may be reduced by enhancing uptake and tissue penetration of the antibodies by modifications such as, for example, lipidation or addition of cell-specific tags.

The compounds or pharmaceutical compositions of the invention can be tested *in vitro*, and then *in vivo* for the desired therapeutic or prophylactic activity, prior to use in humans. For example, *in vitro* assays to demonstrate the therapeutic or prophylactic utility of a compound or pharmaceutical composition include, the effect of a compound on a cell line or a patient tissue sample. The effect of the compound or composition on the cell line and/or tissue sample can be

determined utilizing techniques known to those of skill in the art including, but not limited to, rosette formation assays and cell lysis assays. In accordance with the invention, *in vitro* assays which can be used to determine whether administration of a specific compound is indicated, include *in vitro* cell culture assays in which a patient tissue sample is grown in culture, and exposed to or otherwise administered a compound, and the effect of such compound upon the tissue sample is observed. The effect of such compounds or pharmaceutical compositions can be determined by measuring a change in the level of expression or a change in the expression profile of osteoclast markers.

The invention provides methods of treatment, inhibition and prophylaxis by administration to a subject an effective amount of a compound or pharmaceutical composition of the invention. The compound is substantially purified such that the compound is substantially free from substances that limit its effect or produce undesired side-effects. The subject is preferably an animal, including but not limited to animals such as cows, pigs, horses, chickens, cats, dogs, etc., and is preferably a mammal, and most preferably human.

Various delivery systems are known and can be used to administer a composition of the invention, for example, encapsulation in liposomes (Langer, Science 249:1527-1533 (1990)), microparticles, microcapsules, recombinant cells capable of expressing the compound, receptor-mediated endocytosis (*see, e.g.*, Wu and Wu, J. Biol. Chem. 262:4429-4432 (1987)), construction of a nucleic acid as part of a retroviral or other vector, and the like. Methods of introduction include, but are not limited to, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural, and oral routes. The compounds or compositions may be administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (*e.g.*, oral mucosa, rectal and intestinal mucosa, etc.) and may be administered together with other biologically active agents. Administration can be systemic or local. The pharmaceutical compounds or compositions of the invention can be introduced into the central nervous system by any suitable route, including intraventricular and intrathecal injection. Intraventricular injection can be facilitated by an intraventricular catheter, for example, attached to a reservoir, such as an Ommaya reservoir. Pulmonary administration can also be employed, for example, by use of an inhaler or nebulizer, and formulation with an aerosolizing agent.

The pharmaceutical compounds or compositions of the invention can be administered locally to the area in need of treatment; this can be achieved by, for example, and not by way of limitation, local infusion during surgery, topical application, for example, in conjunction with a wound dressing after surgery, by injection, by means of a catheter, by means of a suppository, or by means of an implant, said implant being of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes, or fibers.

The compound or composition can be delivered in a controlled release system. Furthermore, a controlled release system can be placed in proximity of the therapeutic target, thus requiring only a fraction of the systemic dose (*see, e.g.*, Goodson, in Medical Applications of Controlled Release, *supra*, vol. 2, pp. 115-138 (1984)). In a further embodiment, a pump may be used. In another embodiment, polymeric materials can be used.

Where the compound of the invention is a nucleic acid encoding a protein, the nucleic acid can be administered *in vivo* to promote expression of its mRNA and encoded protein, by constructing it as part of an appropriate nucleic acid expression vector and administering, for example, by use of a retroviral vector, or by direct injection, or by use of microparticle bombardment for example, a gene gun, or coating with lipids or cell-surface receptors or transfecting agents, or by administering it in linkage to a homeobox-like peptide which is known to enter the nucleus (see e.g., Joliot et al., Proc. Nati. Acad Sci. USA 88:1864-1868 (1991)). Alternatively, a nucleic acid can be introduced intracellularly and incorporated within host cell DNA for expression, by homologous recombination.

The compounds or pharmaceutical compositions of the invention can be used for treating a bone resorption disease in an individual by down-regulating (*i.e.*, decreasing or inhibiting) in the individual at least one osteoclast marker or informative gene shown to be expressed, or expressed in increased levels (as compared with a control), in individuals having a bone resorption disorder or at risk for developing a bone resorption disorder. The osteoclast markers are informative genes and are selected from the group consisting of the genes in Figures 1A-1E. Alternatively, the osteoclast markers are MIP-1γ, CCR1, MIP-1α, RANTES, Brn3a, Brn3b, or Brn3c. In addition, the compounds or pharmaceutical compositions of the invention can be used for treating a bone resorption disease in an individual by up-regulating (*i.e.*, increasing or enhancing) in the individual at least one osteoclast marker shown not to be expressed, or expressed at reduced levels (as compared with a control), in individuals having a bone resorption

disorder or at risk for developing a bone resorption disorder. The osteoclast markers are informative genes and are selected from the group consisting of the genes in Figures 2A-2B.

The compounds or pharmaceutical compositions of the invention can also be used for treating a bone generating disease in an individual by up-regulating (*i.e.*, increasing) in the individual at least one osteoclast marker shown to be expressed, or expressed in increased levels in osteoclasts (as compared with a control), in individuals having a bone generating disorder or at risk for developing a bone generating disorder. The osteoclast markers are informative genes and are selected from the group consisting of the genes in Figures 1A-1E. Alternatively, the osteoclast markers are MIP-1γ, CCR1, MIP-1α, RANTES, Brn3a, Brn3b, or Brn3c. Furthermore, the compounds or pharmaceutical compositions of the invention can be used for treating a bone generating disease in an individual by down-regulating (*i.e.*, decreasing or inhibiting) in the individual at least one osteoclast marker shown not to be expressed, or expressed at reduced levels in osteoclasts (as compared with a control), in individuals having a bone generating disorder or at risk for developing a bone generating disorder. The osteoclast markers are informative genes and are selected from the group consisting of the genes in Figures 2A-2B.

The invention further relates to a method of assessing treatment efficacy in an individual having a bone resorption disease, comprising determining the expression level of one or more informative genes at multiple time points, for example, two, three, or more time points during treatment. A treatment can be considered efficacious if the gene expression profile with regard to one or more informative genes tends toward a normal gene expression profile. That is, for example, treatment can be considered efficacious if a gene having increased expression in a disorder (e.g., a bone resorption disease or a bone generating disease) shows reduced expression (i.e., expression tending toward normal expression) or a leveling off of expression as a result of treatment. In addition, treatment can be considered efficacious if a gene having decreased expression in a disorder (e.g., a bone resorption disease or a bone generating disease) shows increased expression (i.e., expression tending toward normal expression) or a leveling off of expression as a result of treatment. For example, in one method, a baseline gene expression profile for the individual can be determined, and repeated gene expression profiles can be determined at time points during treatment. A shift in gene expression profile from a profile correlated with poor treatment outcome to profile correlated with improved treatment outcome is

evidence of an effective therapeutic regimen, while a repeated profile correlated with poor treatment outcome is evidence of an ineffective therapeutic regimen. Figures 1A-1E and 2A-2B provide gene products that are useful in evaluating the efficacy of treatment of a bone resorption disease or a bone generating disease.

A decrease in expression of the one or more osteoclast markers shown to be expressed, or expressed at increased levels in osteoclasts (as compared with a control), in individuals having a bone resorption disease or at risk for developing a bone resorption disease, is indicative that treatment is effective. Alternatively, a lack of a decrease in expression of the one or more osteoclast markers indicates that the treatment is less effective. The osteoclast markers are informative genes that are selected from the group consisting of the genes in Figures 1A-1E. Alternatively, the osteoclast markers are MIP-1γ, CCR1, MIP-1α, RANTES, Brn3a, Brn3b, or Brn3c.

An increase in expression of the one or more osteoclast markers shown not to be expressed, or expressed at reduced levels in osteoclasts (as compared with a control), in individuals having a bone resorption disease or at risk for developing a bone resorption disease, is indicative that treatment is effective. A lack of an increase in expression of the one or more osteoclast markers indicates that the treatment is less effective. The osteoclast markers are informative genes that are selected from the group consisting of the genes in Figures 2A-2B.

The invention also relates to a method of assessing treatment efficacy in an individual having a bone generating disease, comprising determining the expression level of one or more informative genes at multiple time points, for example, two, three, or more time points during treatment. An increase in expression of the one or more osteoclast markers shown to be expressed, or expressed at increased levels in osteoclasts (as compared with a control), in individuals having a bone generating disease or at risk for developing a bone generating disease, is indicative that treatment is effective. A lack of an increase in expression of the one or more osteoclast markers indicates that the treatment is less effective. The osteoclast markers are informative genes that are selected from the group consisting of the genes in Figures 1A-1E.

A decrease in expression of the one or more osteoclast markers shown not to be expressed, or expressed at reduced levels in osteoclasts (as compared with a control), in individuals having a bone generating disease or at risk for developing a bone generating disease, is indicative that treatment is effective. A lack of a decrease in expression of the one or more

osteoclast markers indicates that the treatment is less effective. The osteoclast markers are informative genes that are selected from the group consisting of the genes in Figures 2A-2B.

The invention also relates to a solid substrate, for example, an array, having immobilized thereon a plurality of detection agents that can be used to detect expression and/or biological activity of osteoclast markers, such as informative genes or informative gene products. Examples of detection agents include oligonucleotide probes specific for one or more informative genes and polypeptides (gene expression products) encoded by one or more informative genes. Such arrays can be used to carry out methods for identifying and/or diagnosing bone resorption diseases or bone generating diseases, predicting the likelihood of developing such diseases, identifying compounds for use in treating such diseases, and assessing efficacy of treatment of such diseases, as described herein. The osteoclast markers can be informative genes, which are selected from the group consisting of the genes in Figures 1A-1E and/or 2A-2B. Osteoclast markers also include MIP-1γ, CCR1, MIP-1α, RANTES, Brn3a, Brn3b, or Brn3c. Polypeptide arrays can be used with antibodies or other polypeptides that bind to the polypeptides encoded by the informative genes. Methods and techniques applicable to array (including protein array) synthesis have been described in PCT Application Nos. WO 00/585 16, and WO 99/36760, U.S. Patent Nos. 5,143,854, 5,242,974, 5,252,743, 5,324,633, 5,384,261, 5,405,783, 5,424,186, 5,451,683, 5,482,867, 5,491,074, 5,527,681, 5,550,215, 5,571,639, 15 5,578,832, 5,593,839, 5,599,695, 5,624,711, 5,631,734, 5,795,716, 5,831,070, 5,837,832, 5,856,101, 5,858,659, 5,936,324, 5,968,740, 5,974,164, 5,981,185, 5,981,956, 6,025,601, 6,033,860, 6,040,193, 6,090,555, 6,136,269, 6,269,846 and 6,428,752, which are all incorporated herein by reference in their entirety. Patents that describe synthesis techniques in specific embodiments include U.S. Patent Nos. 5,412,087, 6,147,205, 6,262,216, 6,310,189, 5,889,165, and 5,959,098. Nucleic acid arrays are described in many of the above patents, but the same techniques are applied to polypeptide arrays.

The present invention also contemplates many uses for detection agents attached to solid substrates. These uses include gene expression monitoring, profiling, library screening, genotyping and diagnostics. Gene expression monitoring, and profiling methods can be shown in U.S. Patent Nos. 5,800,992, 6,013,449, 6,020,135, 6,033,860, 6,040,138, 6,177,248 and 6,309,822. Genotyping and uses therefore are shown in U.S. Patent Nos. 5,856,092, 6,300,063,

5,858,659, 6,284,460, 6,361,947, 6,368,799 and 1564.2006-000 6,333,179. Other uses are embodied in U.S. Patents Nos. 5,871,928, 5,902,723, 6,045,996, 5,541,061, and 6,197,506.

The present invention also provides kits that can be used in the above methods. A kit comprises a pharmaceutical composition of the invention in one or more containers. The kit can be a diagnostic kit for use in testing biological samples. The kit can include a control antibody that does not react with the polypeptide of interest in addition to a specific antibody or antigen-binding fragment thereof which binds to the polypeptide (antigen) of the invention being tested for in the biological sample. Such a kit can include a substantially isolated polypeptide antigen comprising an epitope that is specifically immunoreactive with at least one antipolypeptide antigen antibody. Further, such a kit can include a means for detecting the binding of said antibody to the antigen (for example, the antibody may be conjugated to a fluorescent compound such as fluorescein or rhodamine which can be detected by flow cytometry). The kit can include a recombinantly produced or chemically synthesized polypeptide antigen. The polypeptide antigen of the kit can also be attached to a solid support.

The detecting means of the above-described kit includes a solid support to which the polypeptide antigen is attached. The kit can also include a non-attached reporter-labeled anti-human antibody. Binding of the antibody to the polypeptide antigen can be detected by binding of the reporter-labeled antibody. Additionally, the invention includes a diagnostic kit for use in screening serum samples containing antigens of the polypeptide of the invention. The diagnostic kit includes a substantially isolated antibody specifically immunoreactive with polypeptide or polynucleotide antigens, and means for detecting the binding of the polynucleotide or polypeptide antigen to the antibody. The antibody can be attached to a solid support. The antibody can be a monoclonal antibody. The detecting means of the kit can include a second, labeled monoclonal antibody. Alternatively, or in addition, the detecting means can include a labeled, competing antigen.

In one diagnostic configuration, the test serum sample is reacted with a solid phase reagent having a surface-bound antigen obtained by the methods of the present invention. After binding with specific antigen antibody to the reagent and removing unbound serum components by washing, the reagent is reacted with reporter-labeled anti-human antibody to bind reporter to the reagent in proportion to the amount of bound anti-antigen antibody on the solid support. The reagent is washed again to remove unbound labeled antibody, and the amount of reporter

associated with the reagent is determined. The reporter can be an enzyme, for example, which is detected by incubating the solid phase in the presence of a suitable fluorometric, luminescent or calorimetric substrate, as is standard in the art.

The solid surface reagent in the above assay is prepared by known techniques for attaching protein material to solid support material. Suitable solid support materials include, for example and without limitation, polymeric beads, dip sticks, 96-well plate or filter material.

The invention will be further described with reference to the following non-limiting examples. The teaching of all patents, patent applications and all other publications cited herein are incorporated by reference in their entirety.

EXAMPLES

Example 1: Identification of Osteoclast Markers

Genes were identified that are expressed in bone-resorbing cells (osteoclasts) compared to macrophages that have not been stimulated to differentiate into osteoclasts. Genes that were increased in osteoclasts compared to macrophages and genes that were decreased in osteoclasts compared to macrophages, were identified. RNA was prepared from the two cell systems in which osteoclasts differentiate from macrophages. These cell systems included: 1) RAW 264.7 macrophage-like cell line ± RANKL (Receptor Activator of NFκB Ligand); and 2) normal mouse bone marrow macrophages ± M-CSF + RANKL (i.e., the cells received both stimulants or neither stimulant). Unstimulated or stimulated cells were extracted for their content of whole cellular RNA after 4 days (RAW 267.4) or 7 days (normal bone marrow) of stimulation. RNA was purified from each extract, was reversed-transcribed, and was labeled for use as 'mixed' cDNA probes. The four mixed probes (RAW 264.7 - derived osteoclast probes, RAW 264.7 unstimulated probes; normal bone marrow cell osteoclast probes, and RAW 264.7 unstimulated normal marrow probes) were each hybridized to a set of 3 mouse gene arrays (12,000 genes each) (Affymetrix GENECHIP™ Array Murine Genome U74 version 2 set). Genes that were significantly upregulated, as determined using Affymetrix software (in which for increased expression, p values that approach are most significant) during osteoclast differentiation were identified by differences between RANKL-stimulated and unstimulated probes. Genes that showed significant up-regulation in both cell systems were tabulated. Approximately 280 genes showed increased expression in the macrophages induced to differentiate into osteoclasts

compared to their undifferentiated counterparts (listed in Figures 1A-1E) and are thus associated with the osteoclast phenotype. In addition, approximately 79 genes were decreased in the macrophages induced to differentiate into osteoclasts compared to their undifferentiated counterparts (listed in Figures 2A-2B). These down-regulated genes (in which for decreased expression, p values that approach 1 are most significant) are also associated with the osteoclast bone resorbing phenotype.

While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the scope of the invention encompassed by the appended claims.